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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :		(11) International Publication Number: WO 00/62765
A61K 31/00	A2	

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(81) Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
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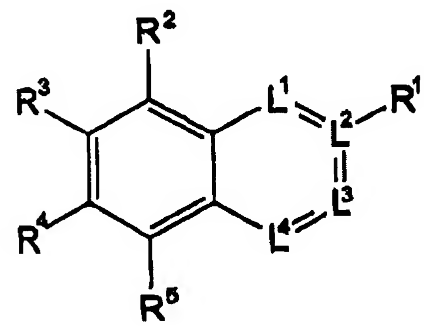
(54) Title: ESTROGEN RECEPTOR-β LIGANDS

(57) Abstract

A method for treating a disease associated with the estrogen receptor-β, comprising the step of administering a therapeutically - effective amount of a compound that satisfies the equation: $(K_{i\alpha A}/K_{i\beta A})/(K_{i\alpha B}/K_{i\beta B})>1$, optionally having the general structure (I).



(I)

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ESTROGEN RECEPTOR-β LIGANDS

Technical Field

The present invention is directed to a series of ligands. and more particularly to estrogen receptor-β ligands which have better selectivity than estrogen for the estrogen receptor-β over the estrogen receptor–α. as well as to methods for their production and use in the treatment of diseases related to the estrogen receptor–β. specifically. Alzheimer's disease. anxiety disorders. depressive disorders. osteoporosis. cardiovascular disease. rheumatoid arthritis. or prostate cancer.

Background

Estrogen-replacement therapy ("ERT") reduces the incidence of Alzheimer's disease and improves cognitive function in Alzheimer's disease patients (Nikolov *et al.* Drugs of Today, 34(11), 927-933 (1998)). ERT also exhibits beneficial effects in osteoporosis and cardiovascular disease, and may have anxiolytic and anti-depressant therapeutic properties. However, ERT shows detrimental uterine and breast side effects that limit its use.

The beneficial effects of ERT in post-menopausal human women is echoed by beneficial effects of estrogen in models relevant to cognitive function, anxiety, depression, bone loss. and cardiovascular damage in ovariectomized rats. Estrogen also produces uterine and breast hypertrophy in animal models reminiscent of its mitogenic effects on these tissues in humans.

The beneficial effects of ERT in post-menopausal human women is echoed by beneficial effects of estrogen in models relevant to cognitive function, anxiety. depression, bone loss. and cardiovascular damage in ovariectomized rats. Specifically, experimental studies have demonstrated that estrogen effects the central nervous system ("CNS") by increasing cholinergic function, increasing neurotrophin / neurotrophin receptor expression, altering amyloid precursor protein processing, providing neuroprotection against a variety of insults. and increasing glutamatergic synaptic transmission, among other effects. The overall CNS profile of estrogen effects in pre-clinical studies is consistent with its clinical utility in improving cognitive function and delaying Alzheimer's disease progression. Estrogen also produces mitogenic effects in uterine and breast tissue indicative of its detrimental side effects on these tissues in humans.

The estrogen receptor ("ER") in humans, rats, and mice exists as two subtypes, ER-α and ER-β, which share about a 50% identity in the ligand-binding domain (Kuiper *et al.* Endocrinology 139(10) 4252-4263 (1998)). The difference in the identity of the subtypes accounts for the fact that some small compounds have been shown to bind preferentially to one subtype over the other (Kuiper *et al.*).

In rats, ER-β is strongly expressed in brain, bone and vascular epithelium, but weakly expressed in uterus and breast, relative to ER-α. Furthermore, ER-α knockout (ERKO-α) mice are sterile and exhibit little or no evidence of hormone responsiveness of reproductive tissues. In contrast, ER-β knockout (ERKO-β) mice are fertile, and exhibit normal development and function of breast and uterine tissue. These observations suggest that selectively targeting ER-β over ER-α could confer beneficial effects in several important human diseases, such as Alzheimer's disease, anxiety disorders, depressive disorders, osteoporosis, and cardiovascular disease without the liability of reproductive system side effects. Selective effects on ER-β-expressing tissues (CNS, bone, etc.) over uterus and breast could be achieved by agents that selectively interact with ER-β over ER-α.

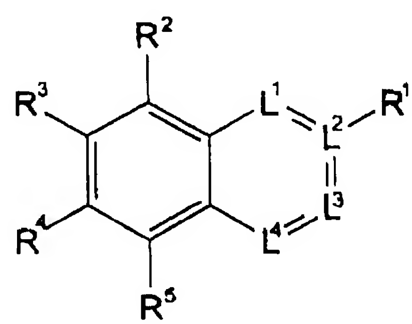
It is a purpose of this invention to identify ER-β-selective ligands that are useful in treating diseases in which ERT has therapeutic benefits.

It is another purpose of this invention to identify ER-β-selective ligands that mimic the beneficial effects of ERT on brain, bone and cardiovascular function.

It is another purpose of this invention to identify ER-β-selective ligands that increase cognitive function and delay Alzheimer's disease progression.

Summary of the Invention

This present invention is directed to the use of compounds having the generic structure:



as ER-β-selective ligands, which mimic ERT, but lack undesirable side effects of ERT. These compounds particularly satisfy the formula:

$(K_{i\alpha A}/K_{i\beta A})/(K_{i\alpha E}/K_{i\beta E}) > 1$,

preferably:

$(K_{i\alpha A}/K_{i\beta A})/(K_{i\alpha E}/K_{i\beta E}) > 30$,

more preferably:

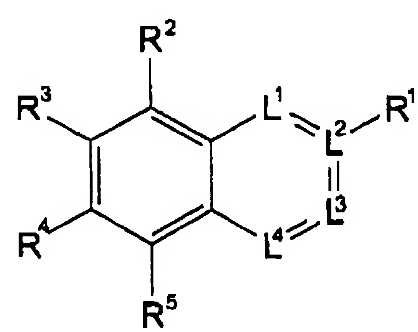
$(K_{i\alpha A}/K_{i\beta A})/(K_{i\alpha E}/K_{i\beta E}) > 100$,

wherein $K_{i\alpha A}$ is the K_i value for the ligand in ER-α; $K_{i\beta A}$ is the Ki value for the ligand in ER-β; $K_{i\alpha E}$ is the K_i value for estrogen in ER-α; and $K_{i\beta E}$ is the K_i value for estrogen in ER-β.

Detailed Description of the Invention

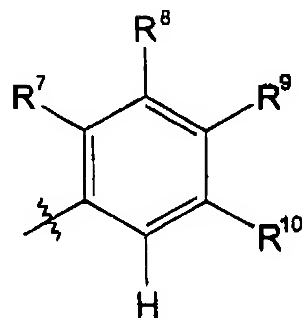
The instant invention involves a method for treating a disease associated with the estrogen receptor-β, comprising the step of administering a therapeutically-effective amount of a compound that satisfies the equation $(K_{i\alpha A}/Ki_{\beta A})/(K_{i\alpha E}/K_{i\beta E}) > 1$, wherein $K_{i\alpha A}$ is the K_i value for the agonist in ER-α; $K_{i\beta A}$ is the K_i value for the agonist in ER-β; $K_{i\alpha E}$ is the K_i value for estrogen in ER-α; and $K_{i\beta E}$ is the K_i value for estrogen in ER-β. Preferably, the compound satisfies the equation $(K_{i\alpha A}/Ki_{\beta A})/(K_{i\alpha E}/K_{i\beta E}) > 100$. Preferred deseases associated with the estrogen receptor-β are selected from Alzheimer's disease, anxiety disorders, depressive disorders, osteoporosis, cardiovascular disease, rheumatoid arthritis and prostate cancer. More preferably, the diseases are Alzheimer's disease or depressive disorders.

The compounds of the instant invention are ER-β-selective ligands of the structure:

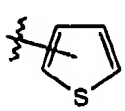
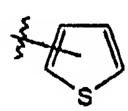


In this structure L^1 is -C(=O)-, =C(R^6)-, -CH(R^6)-, O, S, or NR^a, preferably -C(=O)-, =C(R^6)-, -CH(R^6)- or O; L^2 is =C- or -CH-; L^3 is =C(R^6)-, -CH(R^6)- or -C(=O)-; and L^4 is -C(=O)-, CH_2, O, S, or NR^a, preferably -C(=O)-, CH_2 or O, provided that when L^1 is -C(=O)-, L^4 is CH_2, O, S, or NR^a; when L^4 is -C(=O)-, L^1 is CH_2, O, S, or NR^a; and when L^3 is -C(=O)-, L^1 is =C(R^6)- or -CH(R^6)-, and L^4 is O or NR^a. Additionally, when L^1 is =C(R^6)-, L^2 is =C-; when L^1 is -CH(R^6)-, L^2 is -CH-; when L^3 is =C(R^6)-, L^2 is =C-; and when L^3 is -CH(R^6)-, L^2 is -CH-. ═ represents a single bond or double bond, depending upon the hybridization of L^1-L^4. The structures for L^2 show only three bonds because the fourth bond is a single bond to R^1.

R^1 is attached via a single bond to L^2, and is phenyl, substituted phenyl, Het, or substituted Het, as defined below. R^1 is preferably:



wherein: R^7 is H, Cl, or methyl; R^8 is Br, Cl, F, R^a, OR^a, or allyl; R^9 is H, OH, NH_2, Br, Cl; and R^{10} is H or methyl; or R^8 and R^9 may combine to be -OCH_2O-, forming a secondary 5-membered ring structure exterior to the phenyl group; or R^1 is a substituted or unsubstituted heterocyclic substituent having the following structure:

 ; more preferably unsubstituted  .

R^2, R^3, R^4, and R^5 are each, independently, -R^a, -OR^a, -SR^a, -NR^aR^a, -NC(=O)R^a, -NS(=O)R^a, -NS(=O)$_2R^a$, halogen, cyano, -CF_3, -CO_2R^a, -C(=O)R^a, -C(=O)NHR^a, nitro, -S(=O)R^a, or -S(=O)$_2R^a$, and is preferably R^a, OR^a, $NR^a{}_2$, NC(=O)R^a, CF_3, or halogen, preferably, hydrogen, hydroxyl or methyl.

R^6 is R^a, phenyl or CF_3.

R^a is, independently, at each occurrence, H or (C_1-C_5)alkyl.

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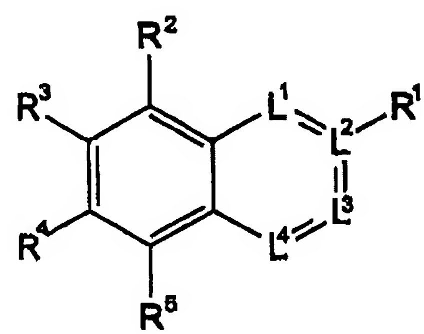

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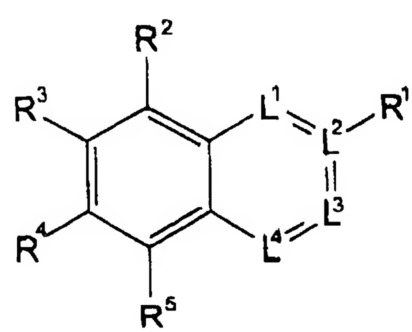

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wherein $K_{i\alpha A}$ is the K_i value for the ligand in ER-α; $K_{i\beta A}$ is the Ki value for the ligand in ER-β; $K_{i\alpha E}$ is the K_i value for estrogen in ER-α; and $K_{i\beta E}$ is the K_i value for estrogen in ER-β.

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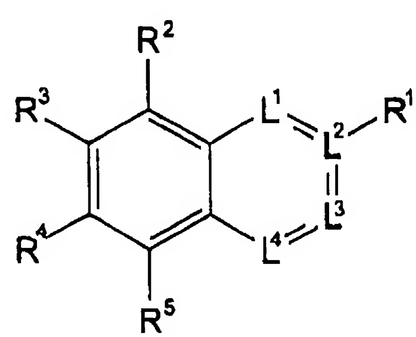

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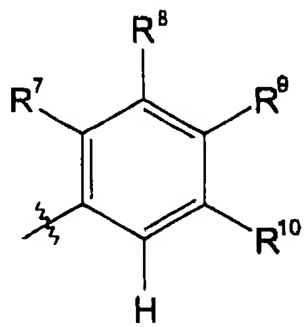
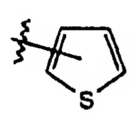
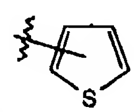


wherein: R^7 is H, Cl, or methyl; R^8 is Br, Cl, F, R^a, OR^a, or allyl; R^9 is H, OH, NH_2, Br, Cl; and R^{10} is H or methyl; or R^8 and R^9 may combine to be $-OCH_2O-$, forming a secondary 5-membered ring structure exterior to the phenyl group; or R^1 is a substituted or unsubstituted heterocyclic substituent having the following structure:

 : more preferably unsubstituted  .

R^2, R^3, R^4, and R^5 are each, independently, $-R^a$, $-OR^a$, $-SR^a$, $-NR^aR^a$, $-NC(=O)R^a$, $-NS(=O)R^a$, $-NS(=O)_2R^a$, halogen, cyano, $-CF_3$, $-CO_2R^a$, $-C(=O)R^a$, $-C(=O)NHR^a$, nitro, $-S(=O)R^a$, or $-S(=O)_2R^a$, and is preferably R^a, OR^a, NR^a_2, $NC(=O)R^a$, CF_3, or halogen, preferably, hydrogen, hydroxyl or methyl.

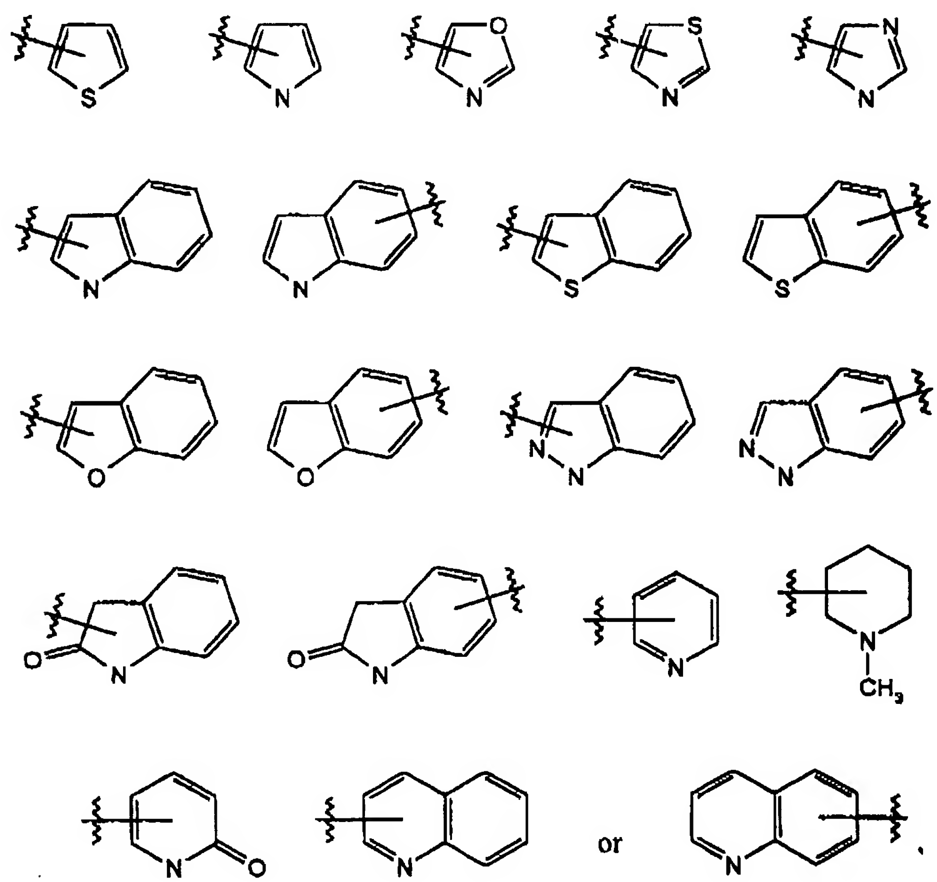
R^6 is R^a, phenyl or CF_3.

R^a is, independently, at each occurrence, H or (C_1-C_5)alkyl.

When L^1 is -C(=O)-. and R^2 is hydroxy or hydrogen. and R^3 is hydrogen, and R^4 is hydroxy. and R^5 is hydrogen, and R^6 is hydrogen, then R^1 is not para-phenol.

For purposes of this invention, "substituted" when used to modify a phenyl or a heteroatomic ring means such a ring substituted at one or more positions. independently, with $-R^a$, $-OR^a$, $-SR^a$, $-NR^aR^a$, $-NC(=O)R^a$, $-NS(=O)R^a$, $-NS(=O)_2R^a$, halogen. cyano, $-CF_3$, $-CO_2R^a$, $-C(=O)R^a$, $-C(=O)NHR^a$, nitro, $-S(=O)R^a$, or $-S(=O)_2R^a$.

Also, for purposes of this invention, "Het" means a substituted or unsubstituted one- or two-ring heterocycle selected from the following:



wherein the crossed bond represents that the heterocycle may be attached at any available position on the ring that it crosses.

Estrogen Receptor Binding Measurements

The ability of a compound to bind to ER was measured by its ability to compete for binding with the radio-labeled estrogen. [^{125}I]-16α–iodo–3,17β–estradiol (NEN. Cat.#NEX-144). The radio-ligand is hereafter referred to as [^{125}I]-estradiol.

ER-β (Gen Bank Accession #X99101) or ER-α (Gen Bank Accession #M12674) cDNAs were cloned into the expression vector pSG5 (Stratagene). transformed into *e. coli* strain DHαF'. and purified using anion-exchange resin columns (Qiagen Cat.#12125). Receptor protein was prepared by *in vitro* transcription and translation of these plasmids using the TNT T7 Quick-Coupled reticulocyte lysate system (Promega Cat.#L1170). Reticulocyte lysate (12.5 mL) was incubated for 90 min at 30 °C with 312.5 μg of ER-α and 625 μg of ER-β plasmids. Programmed lysate was then aliquotted and stored frozen at -80 °C.

Compounds were tested in duplicate at half-log concentrations ranging from 10 pM to 3 μM. Compounds were prepared as 1 mM stocks in DMSO, then diluted in the binding-assay buffer (in mM: 20 HEPES. 150 NaCl, 1 EDTA, 6 monothioglycerol and 10 Na_2MoO_4; 10% wt/vol glycerol, and pH = 7.9) to a series of three-fold concentrated, 20 μL aliquots in a 96-well plate. Receptor aliquots were thawed on ice. and appropriately diluted (see below) in binding assay buffer. Diluted receptor (30 μL/each) was added to each well. [^{125}I]-estradiol was diluted from the manufacturer's ethanol stock solution to a 900 pM working solution in binding-assay buffer. The final assay volume was 60 μL, consisting of 20 μL of a compound according to the instant invention, 30 μL of programmed reticulocyte lysate, and 10 μL of 900 pM [^{125}I]-estradiol. The final concentration of [^{125}I]-estradiol was 150 pM. Plates containing the final assay mixture were mixed on a shaker for 2 min and incubated overnight (~16 h) at 4 °C.

Receptor-bound and unbound radioligand was separated by filtration over sephadex columns. Columns (45 μL bed volume) were prepared by adding dry column media (Pharmacia Cat#G-25) to 96-well column templates (Millipore MultiScreen Plates Cat#MAHVN4510). Columns were then saturated with 300 μL of binding-assay buffer and stored at 4 °C. Prior to use, stored columns were spun for 10 minutes at 2000 RPM. then washed twice with 200 μL of fresh binding buffer. The binding-assay mixtures (50 μL/each) were then applied to the columns, and an additional elution volume of 35 μL was immediately applied to the column. Receptor-bound radioligand was then eluted from the column by

centrifugation for 10 minutes at 2000 RPM. A scintillation cocktail (145 μL) was added to the eluted radioligand/receptor complex, and radio-label was measured by liquid scintillation counting.

Non-specific binding was defined by competition with 150 nM diethylstilbesterol (DES). Binding affinities are expressed as K_i, calculated using the Cheng-Prushoff formula according to IC_{50} values generated by fitting the relationship of concentration to percent specific binding (SB) with the following equation:

$$\% SB = \text{Maximum} - (\text{Maximum} - \text{Minimum})/(1+10^{(\log IC50 - \log[\text{Compound}])})$$

In this assay, standard estrogen receptor ligands estradiol and DES were detected as high-affinity (K_i<1 nM), non-selective ligands of ER-β and ER-α.

The volume of receptor-programmed reticulocyte lysate to be added to the binding assay was determined independently from two measurements made on each batch of receptor prepared. First, K_is were determined for standard compounds using a series of dilutions of the receptor preparation. Scatchard analysis of ligand binding affinity was performed at the receptor dilutions that produced reported K_is for these compounds and an acceptable signal:noise ratio (~10). These experiments indicated a K_D for $[^{125}I]$-estradiol of 0.1-1 nM, and a B_{max} of 5-30 pmol.

Administration and Use

Compounds of the present invention are shown to have high selectivity for ER-β over ER-α, and may possess agonist activity on ER-β without undesired uterine effects. Thus, these compounds, and compositions containing them, may be used as therapeutic agents in the treatment of various CNS diseases related to ER-β, such as, for example, Alzheimer's disease.

The present invention also provides compositions comprising an effective amount of compounds of the present invention, including the nontoxic addition salts, amides and esters thereof, which may, serve to provide the above-recited therapeutic benefits. Such compositions may also be provided together with physiologically-tolerable liquid, gel or solid diluents, adjuvants and excipients. The compounds of the present invention may also be combined with other compounds known to be used as therapeutic agents for the above or other indications.

These compounds and compositions may be administered by qualified health care professionals to humans in a manner similar to other therapeutic agents and, additionally, to

other mammals for veterinary use, such as with domestic animals. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions: solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified. The active ingredient is often mixed with diluents or excipients which are physiologically tolerable and compatible with the active ingredient. Suitable diluents and excipients are, for example, water, saline, dextrose, glycerol, or the like, and combinations thereof. In addition, if desired the compositions may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, stabilizing or pH-buffering agents, and the like.

The compositions are conventionally administered parenterally, by injection, for example, either subcutaneously or intravenously. Additional formulations which are suitable for other modes of administration include suppositories, intranasal aerosols, and, in some cases, oral formulations. For suppositories, traditional binders and excipients may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained-release formulations, or powders.

The present compounds may be formulated into the compositions as neutral or salt forms. Pharmaceutically-acceptable nontoxic salts include the acid addition salts (formed with the free amino groups) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or organic acids such as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

In addition to the compounds of the present invention that display ER-β activity, compounds of the present invention can also be employed as intermediates in the synthesis of such useful compounds.

Synthesis

Compounds within the scope of the present invention may be synthesized chemically by means well known in the art. The following Examples are meant to show general synthetic schemes. which may be used to produce many different variations by employing various commercially-available starting materials. These Examples are meant only as guides on how to make some compounds within the scope of the invention. and should not be interpreted as limiting the scope of the invention.

Examples

Example 1 (Route A)

-(3-Bromo-4-hydroxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one

1,3,5-Trihydroxybenzaldehyde (1.01 g. 6.25 mmol) and 3-bromo-4-hydroxyphenylacetic acid (1.44 g, 6.25 mmol) were suspended in $POCl_3$ (4 mL). After 1 min, an exothermic reaction occured. The mixture was allowed to cool to room temperature. Zinc chloride (1M ether solution, 4.7 mmol) was added and the mixture was heated at 75 °C for 1 h. After cooling, the mixture was partitioned in ethyl acetate and 1M aqueous HCl. The organic layer was washed with brine and dried with $MgSO_4$. Purification on silica gel (MeOH/dichloromethane, gradient) afforded 1-(2,4,6-trihydroxyphenyl)-2-(3-bromo-4-hydroxyphenyl)ethanone (390 mg) as a tan solid.

To 1-(2,4,6-trihydroxyphenyl)-2-(3-bromo-4-hydroxyphenyl)ethanone (370 mg) in DMF (5 mL) under nitrogen was added BF_3-Et_2O (0.83 mL, 6.55 mmol) dropwise, followed by methanesulfonyl chloride (0.507 mL, 6.55 mmol). The mixture was stirred at room temperature for 10 min and heated at 55 °C for 30 min. After cooling, the mixture was partitioned in ethyl acetate / 1M aqueous HCl. The organic layer was washed with 1M HCl and brine. and purified by C_{18} HPLC to give the title compound (55 mg).

Example 2 (Compound No. 28; Route B)

3-(4-hydroxyphenyl)-7-hydroxy-4-methylcoumarin

A solution of 2.4-dihydroxyacetophenone (1.1 g, 7.24 mmol). 4-hydroxyphenylacetic acid (1.45 g, 9.5 mmol) and potassium acetate (0.9 g, 9.2 mmol) in acetic anhydride (10 mL) was heated under reflux for 18 h. After cooling. the mixture was poured into ice and water. The solid was filtered, washed with ether and dried under vacuum to give 3-(4-acetoxyphenyl)-7-acetoxy-4-methylcoumarin (1.83 g).

A suspension of 3-(4-acetoxyphenyl)-7-acetoxy-4-methylcoumarin (500 mg) in THF (10 mL) and 1N aqueous sodium hydroxide (10 mL) was stirred for 1 h. The mixture is acidified to pH = 1 with concentrated HCl and extracted with EtOAc / water. The organic layer was washed with brine and dried over $MgSO_4$. Evaporation of the solvent and trituration of the residue with ether gave the title compound (140 mg).

The HPLC conditions (HPLC 4.6 x 250 mm C_{18} 5 µm Vydax 218TP54 column, flow rate: 1.5 mL/min. acetonitrile/water 0.1% TFA linear gradient from 10:90 to 50:50 over 30 min. UV detection: 254 nm) are referred as conditions A.

The HPLC conditions (HPLC 2.1 x 30 mm C_{18} 3.5 µm Zorbax Rapid Resolution column, flow rate: 0.7 mL/min. water - 0.05% TFA for 0.5 min, then 90% aqueous acetonitrile/water 0.05% TFA linear gradient from 0:100 to 80:20 over 9.5 min, UV detection) are referred as conditions B.

The following compounds were prepared according to these routes, using the relevant starting materials.

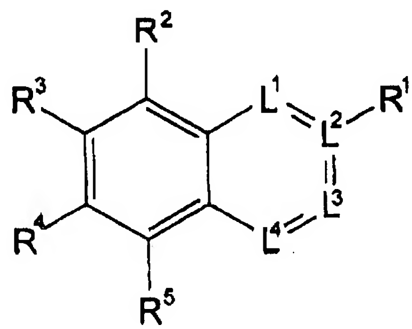


Table 1.

No.	L^1	L^2	L^3	L^4	R^1
1	C(=O)	=C-	=CR^6-	O	3,4-dihydroxyphenyl
2	C(=O)	=C-	=CR^6-	O	2-Cl-4-hydroxyphenyl
3	C(=O)	=C-	=CR^6-	O	2-Me-4-hydroxyphenyl
4	C(=O)	=C-	=CR^6-	O	3-F-4-hydroxyphenyl
5	C(=O)	=C-	=CR^6-	O	3-Cl-4-hydroxyphenyl
6	C(=O)	=C-	=CR^6-	O	3-Br-4-hydroxyphenyl
7	C(=O)	=C-	=CR^6-	O	3-allyl-4-hydroxyphenyl
8	C(=O)	=C-	=CR^6-	O	3-Pr-4-hydroxyphenyl

No.	L^1	L^2	L^3	L^4	R^1
9	C(=O)	=C-	$=CR^6$-	O	3-methoxy-4-hydroxyphenyl
10	C(=O)	=C-	$=CR^6$-	O	3,5-diMe-4-hydroxyphenyl
11	C(=O)	=C-	$=CR^6$-	O	4-fluorophenyl
12	C(=O)	=C-	$=CR^6$-	O	3,4-(OCH_2O)phenyl
13	C(=O)	=C-	$=CR^6$-	O	4-aminophenyl
14	C(=O)	=C-	$=CR^6$-	O	2-naphthyl
15	C(=O)	=C-	$=CR^6$-	O	3-hydroxyphenyl
16	C(=O)	=C-	$=CR^6$-	O	2-hydroxyphenyl
17	C(=O)	=C-	$=CR^6$-	O	2-thiophene
18	C(=O)	=C-	$=CR^6$-	O	3-thiophene
19	C(=O)	=C-	$=CR^6$-	O	2-quinolinyl
20	C(=O)	=C-	$=CR^6$-	O	4-bromophenyl
21	C(=O)	=C-	$=CR^6$-	O	4-chlorophenyl
22	C(=O)	=C-	$=CR^6$-	O	4-hydroxyphenyl
23	C(=O)	=C-	$=CR^6$-	O	4-hydroxyphenyl
24	C(=O)	=C-	$=CR^6$-	O	3-F-4-hydroxyphenyl
25	C(=O)	=C-	$=CR^6$-	O	4-hydroxyphenyl
26	C(=O)	-CH-	$-CHR^6$-	O	4-hydroxyphenyl
27	C(=O)	-CH-	$-CHR^6$-	CH_2	4-hydroxyphenyl
28	$=CR^6$-	=C-	C(=O)	O	4-hydroxyphenyl
29	$=CR^6$-	=C-	C(=O)	O	4-hydroxyphenyl
30	$=CR^6$-	=C-	C(=O)	O	4-hydroxyphenyl
31	$=CR^6$-	=C-	C(=O)	O	2-thiophene
32	C(=O)	=C-	$=CR^6$-	O	4-hydroxyphenyl
33	C(=O)	=C-	$=CR^6$-	O	2-F-phenyl
34	C(=O)	=C-	$=CR^6$-	O	phenyl
35	C(=O)	=C-	$=CR^6$-	O	phenyl
36	O	=C-	$=CR^6$-	C(=O)	4-hydroxyphenyl

No.	L^1	L^2	L^3	L^4	R^1
37	CH_2	-CH-	$-CHR^6-$	C(=O)	4-hydroxyphenyl
38	$=CR^6-$	=C-	C(=O)	O	4-hydroxyphenyl
39	$=CR^6-$	=C-	C(=O)	O	4-hydroxyphenyl
40	$=CR^6-$	=C-	C(=O)	O	4-hydroxyphenyl
41	$=CR^6-$	=C-	C(=O)	O	4-hydroxyphenyl
42	$=CR^6-$	=C-	C(=O)	O	4-Cl-phenyl
43	$=CR^6-$	=C-	C(=O)	O	4-hydroxyphenyl
44	C(=O)	=C-	$=CR^6-$	O	4-isopropoxyphenyl
45	C(=O)	-CH-	$-CHR^6-$	CH_2	3-Br-phenyl
46	CH_2	-CH-	$-CHR^6-$	O	4-hydroxyphenyl

(Continuation of Table 1)

No.	R^2	R^3	R^4	R^5	R^6
1	OH	H	OH	H	H
2	OH	H	OH	H	H
3	OH	H	OH	H	H
4	OH	H	OH	H	H
5	OH	H	OH	H	H
6	OH	H	OH	H	H
7	OH	H	OH	H	H
8	OH	H	OH	H	H
9	OH	H	OH	H	H
10	OH	H	OH	H	H
11	OH	H	OH	H	H
12	OH	H	OH	H	H
13	OH	H	OH	H	H
14	OH	H	OH	H	H
15	OH	H	OH	H	H
16	OH	H	OH	H	H

No.	R^2	R^3	R^4	R^5	R^6
17	OH	H	OH	H	H
18	OH	H	OH	H	H
19	OH	H	OH	H	H
20	OH	H	OH	H	H
21	OH	H	OH	H	H
22	OH	H	OMe	H	H
23	Me	H	OH	H	H
24	H	H	OH	H	H
25	H	H	OH	H	CF_3
26	OH	H	OH	H	H
27	OH	H	OH	H	H
28	H	H	OH	H	Me
29	H	H	OH	H	Et
30	H	H	H	H	H
31	H	H	OH	H	H
32	OH	H	OH	OMe	H
33	OH	H	OH	H	H
34	OH	H	OH	H	Ph
35	H	H	OH	H	Ph
36	H	H	OH	H	H
37	H	H	OH	H	H
38	H	H	OH	H	H
39	OH	H	OH	H	H
40	H	H	H	OH	H
41	H	OH	H	H	H
42	H	H	OH	H	Me
43	H	H	OH	Me	Me
44	H	H	OH	H	CF_3
45	H	H	OH	H	H

No.	R^2	R^3	R^4	R^5	R^6
46	H	H	OH	H	H

Table 2. Purification, Properties, and Synthetic Route

No.	HPLC min (method)	MS (MH^+)	ER-β K_i nM	ER-α K_i nM	Synthetic Route
1			2.15	605	*
2	5.76 (B)	305 (^{35}Cl)	0.55	56	A
3	5.41 (B)	285	1.2	61	A
4	5.62 (B)	289	0.5	74	A
5	6.11 (B)	305 (^{35}Cl)	1.2	1100	A
6	25.6 (A)	349 (^{79}Br)	1.25	439	A
7	6.72 (B)	311	3.2	>3000	A
8	7.08 (B)	313	0.75	>3000	A
9			143	>3000	*
10	25.4 (A)	299	25	>3000	A
11	6.93 (B)	273	100	>3000	A
12			22	>3000	*
13			6	>3000	*
14	7.86 (B)	305	150	>3000	A
15	5.39 (B)	271	15	900	A
16	5.68 (B)	271	110	>3000	A
17	^{1}H NMR (DMSO-d_6): 12.59 (s, 1H), 10.99 (s, 1H), 8.88 (s, 1H), 7.63 (m, 2H), 7.14 (m, 1H), 6.44 (s, 1H), 6.27 (s, 1H).		3.3	>3000	A
18	^{1}H NMR (DMSO-d_6): 12.92 (s, 1H), 10.93 (s, 1H), 8.72 (s, 1H), 8.07 (s, 1H), 7.64 (m, 1H), 7.53 (m, 1H), 6.42 (s, 1H), 6.24 (s, 1H).		17	>3000	A
19	5.26 (B)	306	122	>3000	A
20	7.70 (B)	333 (^{79}Br)	25	>3000	A

No.	HPLC min (method)	MS (MH+)	ER-β K_i nM	ER-α K_i nM	Synthetic Route
21	7.55 (B)	289 (^{35}Cl)	42	>3000	A
22			50	>3000	*
23	5.20 (B)	269	0.5	200	A
24	4.91 (B)	273	3.3	>3000	A
25	6.07 (B)	323	10	321	Note a)
26			3.7	1000	*
27	5.43 (B)	271	5.7	3000	Note b)
28	^{1}H NMR (DMSO-d_6): 10.47 (m, 1H), 9.55 (m, 1H), 7.67 (d, 1H), 7.1-6.7 (m, 6H), 2.22 (m, 3H); MS: 269		12	322	B
29	5.57 (B)	283	4	80	B
30	6.01 (B)	239	140	>3000	B
31	^{1}H NMR (DMSO-d_6): 10.68 (s, 1H), 8.44 (s, 1H), 7.75 (m, 1H), 7.60 (m, 2H), 7.16 (m, 1H), 6.87 (dd, 1H), 6.81 (m, 1H); MS: 245		108	>3000	B
32			33	>3000	*
33	^{1}H NMR (DMSO d-6): 12.66 (s, 1H), 10.98 (s, 1H), 8.42 (s, 1H), 7.48 (m, 2H), 7.27 (m, 2H), 6.44 (d, 1H, J= 2.1 Hz), 6.26 (d, 1H, J= 2.1 Hz); MS: 273		50	>3000	A
34			9.5	95	*
35			19	50	*
36			0.33	88	*
37	^{1}H NMR (DMSO d-6): 9.61 (s, 1H), 9.52 (s, 1H), 7.26 (d, 1H, J= 2.7 Hz), 7.21-7.13 (m, 3H), 6.99 (dd, 1H, J= 8.1 Hz, J'= 2.7 Hz), 6.71 (d, 2H, J= 8.4 Hz), 3.26 (m, 1H), 3.07-2.80 (m, 3H), 2.64 (m, 1H); MS: 253 (M-H)$^-$		0.73	75	Note c)

No.	HPLC min (method)	MS (MH^+)	ER-ß K_i nM	ER-α K_i nM	Synthetic Route
38	^{1}H NMR (DMSO d-6): 10.52 (s, 1H), 9.64 (s, 1H), 8.03 (s, 1H), 7.55 (m, 3H), 6.85-6.70 (m, 4H); MS: 255		4.9	220	B
39	^{1}H NMR (DMSO d-6): 10.63 (s, 1H), 10.33 (s, 1H), 9.60 (s, 1H), 7.95 (s, 1H), 7.50 (d, 2H, J= 8.4 Hz), 6.80 (d, 2H, J= 8.4 Hz), 6.28 (s, 1H), 6.22 (s, 1H): MS: 271		79	>3000	B
40	^{1}H NMR (DMSO d-6): 10.18 (s, 1H), 9.73 (s, 1H), 8.08 (s, 1H), 7.60 (d, 2H, J= 8.4 Hz), 7.17 (m, 2H), 7.06 (m, 1H), 6.85 (d, 2H, J= 8.4 Hz); MS: 255		104	>3000	B
41	^{1}H NMR (DMSO d-6): 9.72 (s, 2H), 8.05 (s, 1H), 7.58 (d, 2H, J= 8.4 Hz), 7.25 (d, 1H, J= 8.7 Hz), 7.07 (d, 1H, J= 2.7 Hz), 7.00 (dd, 1H, J= 8.4 Hz, J'= 2.7 Hz), 6.84 (d, 2H, J= 8.4 Hz); MS: 255		4.6	3000	B
42	^{1}H NMR (DMSO d-6): 10.56 (s, 1H), 7.50 (d, 2H, J= 7.8 Hz), 7.42 (d, 1H, J= 8.7 Hz), 7.33 (d, 2H, J= 7.8 Hz), 6.84 (dd, 1H, J= 7.8 Hz, J'= 2.1 Hz), 6.75 (d, 1H, J= 2.1 Hz), 2.21 (s, 3H); MS: 287 (^{35}Cl)		51	>3000	B
43	^{1}H NMR (DMSO d-6): 10.36 (s, 1H), 9.55 (s, 1H), 7.49 (d, 1H, J= 9 Hz), 7.08 (d, 2H, J= 8.7 Hz), 6.87 (d, 1H, J= 9 Hz), 6.81 (d, 2H, J= 8.7 Hz), 2.21 (s, 3H), 2.19 (s, 3H); MS: 283		24	500	B
44	^{1}H NMR (DMSO d-6): 11.11 (s, 1H), 7.93 (d, 1H, J= 8.7 Hz), 7.16 (d, 2H, J= 8.4 Hz), 7.03-6.93 (m, 4H), 4.66 (m, 1H), 1.30 (d, 6H, J= 6Hz); MS: 365		118	3000	Note a)

No.	HPLC min (method)	MS (MH^+)	ER-ß K_i nM	ER-α K_i nM	Synthetic Route
45	^{1}H NMR (DMSO d-6): 10.39 (s, 1H), 7.78 (d, 1H, J= 8.4 Hz), 7.42 (m, 2H), 7.28 (t, 1H, J= 7.8 Hz), 7.19 (d, 1H, J= 7.8 Hz), 6.75 (dd, 1H, J= 8.4 Hz, J'= 2.4 Hz), 6.69 (d, 1H, J= 2.4 Hz), 3.86 (m, 1H), 3.00 (m, 1H), 2.85 (m, 1H), 2.4-2.1 (m, 2H); MS: 317 (^{79}Br)		116	3000	Note b)
46			2	155	*

* compound is commercially available.

Note a): Prepared according to method A; the cyclization step was done using trifluoroacetic anhydride according to J. Med. Chem. 1992, 35, 3519.

Note b): Prepared by cyclization of the corresponding 2,4-diarylbutyric acid with $POCl_3$, and subsequent demethylation of the methoxy ethers according to the method developed in J. Org. Chem. 1946, 11, 34.

Note c): Prepared according to Aust. J. Chem. 1978, 31, 1011.

CLAIMS:

1. A method for treating a disease associated with the estrogen receptor-β, comprising the step of administering a therapeutically-effective amount of a compound that satisfies the equation:

$$(K_{i\alpha A}/Ki_{\beta A})/(K_{i\alpha E}/K_{i\beta E}) > 1,$$

wherein

$K_{i\alpha A}$ is the K_i value for the agonist in ER-α;

$K_{i\beta A}$ is the K_i value for the agonist in ER-β;

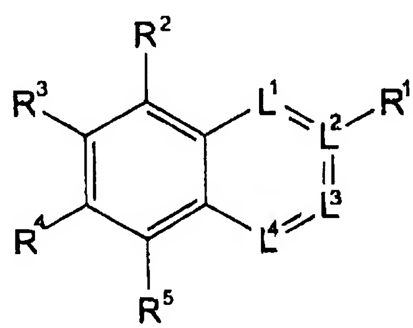
$K_{i\alpha E}$ is the K_i value for estrogen in ER-α; and

$K_{i\beta E}$ is the K_i value for estrogen in ER-β.

2. The method according to Claim 1, wherein the compound satisfies the equation: $(K_{i\alpha A}/Ki_{\beta A})/(K_{i\alpha E}/K_{i\beta E}) > 100$.

3. The method according to Claim 2, wherein the disease to be treated is selected from the group consisting of Alzheimer's disease, anxiety disorders, depressive disorders, osteoporosis, cardiovascular disease, rheumatoid arthritis and prostate cancer.

4. The method according to Claim 3, wherein the compound has the formula:



wherein:

L^1 is -C(=O)-, =C(R^6)-, -CH(R^6)-, O, S, or NR^6;

L^2 is =C- or -CH-;

L^3 is $=C(R^6)$-, $-CH(R^6)$- or $-C(=O)$-;

L^4 is $-C(=O)$-, CH_2, O, S, or NR^a;

wherein:

when L^1 is $-C(=O)$-, L^4 is CH_2, O, S, or NR^a;

when L^4 is $-C(=O)$-, L^1 is CH_2, O, S, or NR^a;

when L^3 is $-C(=O)$-, L^1 is $=C(R^6)$- or $-CH(R^6)$-, and L^4 is O or NR^a

when L^1 is $=C(R^6)$-, L^2 is =C-;

when L^1 is $-CH(R^6)$-, L^2 is -CH-;

when L^3 is $=C(R^6)$-, L^2 is =C-; and

when L^3 is $-CH(R^6)$-, L^2 is -CH-;

R^a is, independently, at each occurrence, H or $(C_1$-$C_5)$alkyl;

R^1 is phenyl, substituted phenyl or Het;

R^2, R^3, R^4 and R^5 are independently selected from the group consisting of $-R^a$, $-OR^a$, $-SR^a$, $-NR^aR^a$, $-NC(=O)R^a$, $-NS(=O)R^a$, $-NS(=O)_2R^a$, halogen, cyano, $-CF_3$, $-CO_2R^a$, $-C(=O)R^a$, $-C(=O)NHR^a$, nitro, $-S(=O)R^a$ and $-S(=O)_2R^a$;

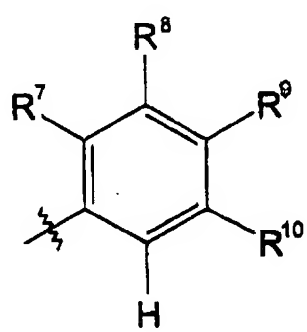
R^6 is H, $(C_1$-$C_5)$alkyl, phenyl or CF_3; and

wherein, when L^1 is $-C(=O)$-, and R^2 is hydroxy or hydrogen, and R^3 is hydrogen, and R^4 is hydroxy, and R^5 is hydrogen, and R^6 is hydrogen then R^1 is not para-phenol; and any pharmaceutically-acceptable salt thereof.

5. The method according to Claim 4, wherein R^1 is Het.

6. The method according to Claim 4, wherein:

R^1 has the structure:



wherein:

R^7 is H, Cl or methyl;

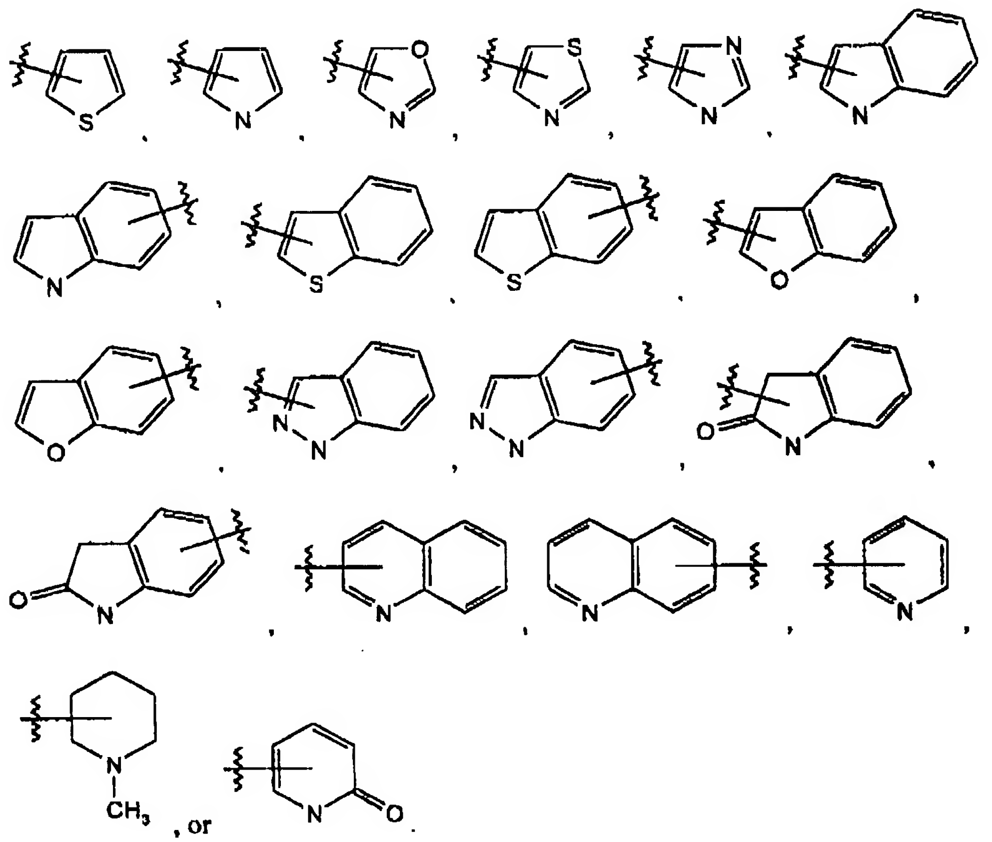
R^8 is Br, Cl, F, R^a, OR^a or allyl;

R^9 is H, OH, NH_2, Br or Cl; and

R^{10} is H or methyl; or

R^8 and R^9 combine to form $-OCH_2O-$; or

R^1 is a substituted or unsubstituted version of one of the following:



7. The method according to any one of Claims 6, wherein the disease is Alzheimer's disease or depressive disorders.

8. The method according to Claim 6 wherein R^2, R^3, R^4 and R^5 are independently selected from the group consisting of R^a, OR^a, NR^a_2, $NC(=O)R^a$, CF_3 and halogen.

9. The method according to Claim 8 wherein:

R^2 is hydroxyl or hydrogen;

R^3 is hydrogen or methyl;

R^4 is hydroxyl or hydrogen; and

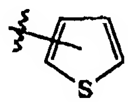
R^5 is hydrogen or hydroxyl.

10. The method according to Claim 8 wherein L^4 is -C(=O)-.

11. The method according to Claim 8 wherein L^3 is -C(=O)-.

12. The method according to Claim 8 wherein L^1 is -C(=O)-.

13. The method according to Claim 9 wherein R^1 is an unsubstituted version of



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Interaction of Phytoestrogens with Estrogen Receptors α and β

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The human estrogen receptor (hER) exists as two subtypes, hER α and hER β, that differ in the C-terminal ligand-binding domain and in the N-terminal transactivation domain. In this study, we investigated the estrogenic activities of soy isoflavones after digestion with enteric bacteria in competition binding assays with hER α or hER β protein, and in a gene expression assay using a yeast system. The estrogenic activities of these isoflavones were also investigated by the growth of MCF-7 breast cancer cells.

Isoflavone glycoside binds weakly to both receptors and estrogen receptor-dependent transcriptional expression is poor. The aglycones bind more strongly to hER β than to hER α. The binding affinities of genistein, dihydrogenistein and equol are comparable to the binding affinity of 17 β-estradiol. Equol induces transcription most strongly with hER α and hER β. The concentration required for maximal gene expression is much higher than expected from the binding affinities of the compounds, and the maximal activity induced by these compounds is about half the activity of 17 β-estradiol. Although genistin binds more weakly to the receptors and induces transcription less than does genistein, it stimulates the growth of MCF-7 cells more strongly than does genistein.

Key words isoflavone; human estrogen receptor (hER) α, β; isoflavone binding to hER; hER-dependent gene expression; hER-dependent MCF-7 cell growth

Estrogens play important hormonal roles in all vertebrates. Animal estrogens are exclusively steroidal compounds, and the principal physiological estrogen in most species is 17 β-estradiol. Many plants produce compounds that possess estrogenic activity in animals and are thus called phytoestrogens.

Among the foods consumed by humans, soybeans contain the highest concentration of isoflavones. These soy isoflavones (*e.g.*, daidzin, genistin and glycitin) may have some health-enhancing properties such as prevention of certain cancers,[1] lowering the risk of cardiovascular diseases,[2] and improvement of bone health.[3] The estrogenic activities of these isoflavones may play an important role in their health-enhancing properties. Soy isoflavones have been reported to bind to estrogen receptors and prevent cell growth in breast cancer cells.[4,5]

We have systematically examined the metabolism of soy isoflavones by enteric bacteria and identified these metabolites.[6]

Two estrogen receptors (ERs) have been identified to date[7,8] and the physiological responses to estrogen are known to be mediated within specific tissues by at least these two receptors. The ERs are a 3A member of the nuclear hormone receptor family and act as ligand-activated nuclear transcription factors.[9]

In this paper, we examined the estrogenic activities of the isoflavone metabolites by (A) their binding to hER α and β, (B) their effect on estrogen receptor-dependent transcriptional expression, and (C) their effect on the growth of MCF-7 cells, which requires estrogen for growth.[10,11]

MATERIALS AND METHODS

Chemicals 17 β-Estradiol, Diethylstilbestrol (DES), Bisphenol A (Bis A) and Nonylphenol (NP) were purchased from Sigma Chemical Co. [2,4,6,7-^{3}H(N)]-17 β-Estradiol (72 Ci/mmol) was purchased from Dai-Ichi Pure Chemicals Co., Ltd. MCF-7 cells were purchased from Dainippon Pharmaceutical Co., Ltd.

Isoflavones Soybean isoflavones were digested by enteric bacteria and the structures of these digests were determined and isolated as reported.[6] The products, which are examined in this paper, are shown in Fig. 1.

Preparation of the Extract of Human Estrogen Receptor α and β hER α cDNA was isolated from pBacPAK9-HEG0 kindly supplied by S. Kato[12] by digestion with *Bam*HI and *Xho*I. hER β cDNA was isolated from pGEX-4T-2-hER β[13] by digestion with *Bam*HI and *Xho*I. These fragments were ligated into the *Bam*HI/*Xho*I sites of the baculovirus donor vector pFastBac 1 (Life Technologies, Gaithersburg, MD, U.S.A.). Recombinant baculoviruses were generated using the BAC-TO-BAC expression system (Life Technologies) in accordance with the manufacturer's instructions. The recombinant baculoviruses were amplified and used to infect Sf21 cells (Clontech, Palo Alto, CA, U.S.A.). Infected cells were incubated at 28 °C and harvested 72 h post infection by centrifugation. The cells were suspended in buffer containing 40 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.2 M KCl, 10% glycerol, 1 mM DTT, and 1 mM PMSF. The extracts were prepared by sonication (10 s×2). The supernatants of the extracts after centrifugation (15000 rpm×10 min) contained *ca.* 6 mg/ml protein and were used as hER α and β. The concentrations of hER α and β were measured using purified hER α and β purchased from Takara Shuzo

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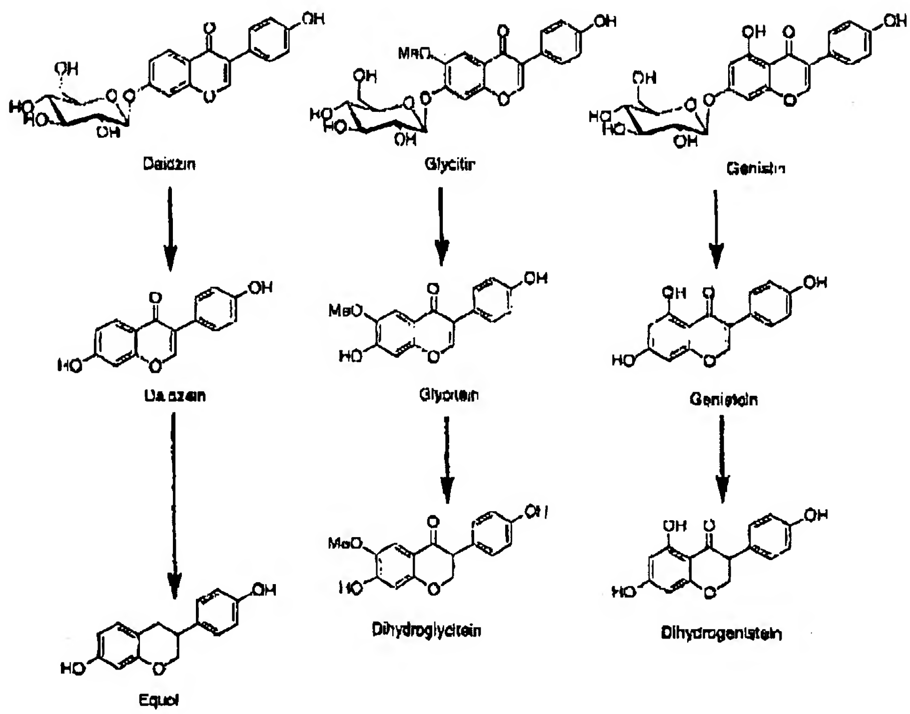

Fig. 1. Structures of Metabolites Obtained by the Digestion of Soy Isoflavones by Enteric Bacteria

Co., Ltd. as a standard. Their concentrations were 0.6% and 0.3% of the total protein, respectively. These receptors were stable at −80 °C for several months.

Competition Assay of Isoflavone Estrogen binding to hER α or β was determined by incubation of 250 μl of reaction mixture of TKE (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 20 mM KCl) containing 5 μl hER α or β with 2.5 pmol [^{3}H]-17 β-estradiol at 0 °C (for 16 h) in the presence of various concentrations of isoflavones. Isoflavones were stable under this incubation condition. Free and bound ligands were separated by addition of an equal volume of dextran-coated charcoal (0.5% activated charcoal and 0.05% dextran) in TKE. Samples were treated for 5 min on ice with periodic mixing and centrifuged at 15000 rpm for 1 min. Aliquots of the supernatant (300 μl) were used for scintillation counting.

Construction of Yeast Strain Carrying Full-Length hER α or β *Saccharomyces cerevisiae* Y190 (*MATa, ura3-52, his3-D200, ade2-101, trp1-901, leu2-3, 112, gal4Dgal80D, URA3::: GAL-lacZ, cyhr2, LYS2::: GAL-HIS3*) which carries pGBT9-ratER and pGAD424-*hTIF2* was kindly supplied by Nishikawa.[14] We substituted hER α or hER β for ratER. pGBT9-ratERLBD was digested with *Eco*RI, and then the cleaved open ends were treated with S1 nuclease. The digest was further digested with *Bam*HI followed by treatment with a Klenow fragment and closed by ligation. The plasmid was redigested with *Bam*HI and *Sal*I. pGBT9-hER α or pGBT9-hER β was prepared by inserting a fragment containing full-length hER α or hER β obtained by the digestion of pBacPAK9 HEG0 or pGEX4T-2-ER β at *Bam*HI and *Xho*I sites into the sites obtained by the digestion of pGBT9 by *Bam*HI and *Sal*I (Fig. 2).

Estrogen Receptor-Dependent Transcriptional Expression Induced by Isoflavone The effect of isoflavones on the estrogen receptor-dependent transcription of β-galactosidase in yeast was examined following the methods described by Nishikawa and his colleages.[14] Yeast cells carrying hER α or β were constructed as described by these same authors.[14]

Growth of MCF-7 Cells MCF-7 cells were grown in phenol red-free DMEM (Gibco BRL, Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, U.S.A.), penicillin and streptomycin (Gibco BRL, Grand Island, NY, U.S.A.). Cells were grown as a monolayer under these conditions. Cells were harvested as needed for use in experimental trials by trypsinization (0.05% trypsin, 0.53 mM EDTA-4Na; Gibco) to yield a suspension of cells for plating in 96-well tissue-culture plates (Falcon; Becton Dickinson, Franklin Lakes, NJ, U.S.A.). Cells were plated at a concentration of 2×10^4 cells/well in phenol red-free DMEM supplemented with 5% heat-inactivated dextran charcoal-stripped FBS (Hyclone, Logan, UT, U.S.A.) for 24 h prior to the addition of phytoestrogen. The growth of the cells was measured by a sulforhodamine B (SRB) assay[21] after 5 d incubation.

RESULTS

Estrogenic Activities of Isoflavones DES, Bis A, and NP are known to bind ER, induce transcription and stimulate the growth of MCF-7 cells.[16—18] We used these compounds as controls. The results are shown in Fig. 3. DES binds both hER α and β almost as strongly as 17 β-estradiol. Bis A and NP bind hER β better than α. The concentration required to bind 50% is about 10^4 times greater for Bis A or NP than it is for 17 β-estradiol. Tamoxifen which is known as an estrogen

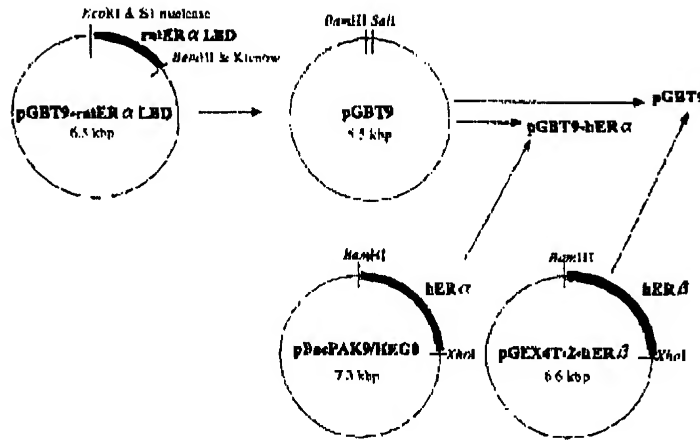

Fig. 2. Construction of pGBT9-hER α and pGBT9-hER β

hER α fragment was obtained by the digestion of pBacPAK9/HEG0 by *Bam*HI and *Xho*I. This fragment was ligated with the fragment obtained by digesting pGBT9 by *Bam*HI and *Sal*I to prepare pGBT9-hER α. The hER β fragment was obtained by digesting pGEX4T-2-hER β by *Bam*HI and *Xho*I. This fragment was ligated with the fragment obtained by digesting pGBT9 by *Bam*HI and *Sal*I to prepare pGBT9-hER β.

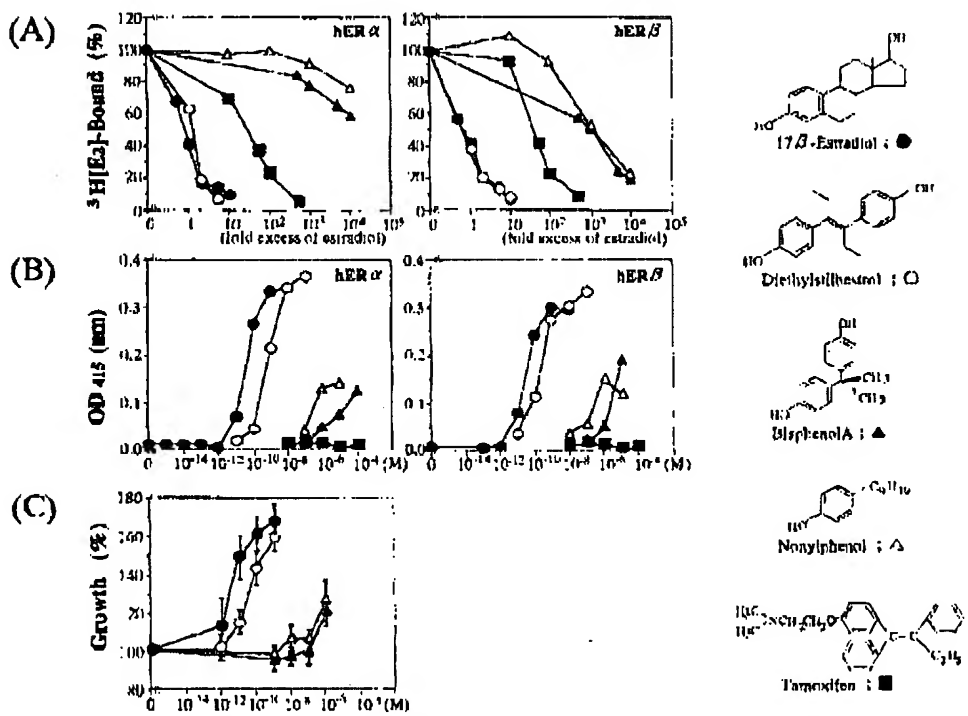

Fig. 3. Assays of (A) Binding of Estrogenic Compounds to hERs, (B) Estrogen Receptor-Dependent Transcriptional Expression, and (C) Growth of MCF-7 Cells

●, 17 β-estradiol; ○, DES; ▲, Bis A; △, NP; ■, tamoxifen. Binding to the ERs was examined by competition as described in Materials and Methods. Estrogen receptor-dependent β-galactosidase induction was measured by the increase of OD_{415nm} by o-nitrophenol presence by the digestion of o-nitrophenyl-β-D-galactoside. The growth of MCF-7 cells was measured by an SRB assay and the results are shown as percent increase. Although we have obtained fairly constant results concerning the binding to the ERs and the induction of β-galactosidase, the results of the growth of MCF-7 cells varied. We have done at least 10 experiments of the growth of MCF-7 cells with each compound and the average is shown. The bar at each point is the standard deviation.

antagonist[19] binds both hER α and β but does not induce transcription. Testosterone did not bind to these receptors confirming their specificity. We also confirmed that 17 β-estradiol did not bind to the cell extract prepared from the cells infected by vector baculovirus (data not shown).

In the experiments of estrogen receptor-dependent transcriptional expression, DES induces as efficiently as 17 β-estradiol. The concentration required for the maximal induction is about 10^4 times greater for Bis A or NP than it is for 17 β-estradiol. These drugs stimulate the growth of MCF-7 cells as shown in Fig. 3. We confirmed that 17 β-estradiol did not induce transcription with the cells carrying vector plasmid pGBT9 (data not shown).

The estrogenic activities of the isoflavone derivatives

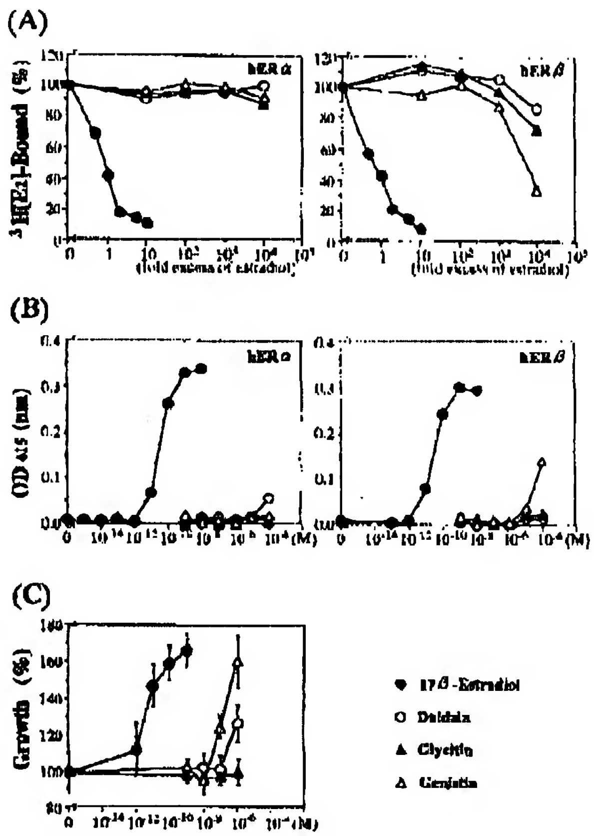

Fig. 4. The Same Assay as Fig. 3 with Daidzin (○), Glycitin (▲), and Genistin (△)

17 β-Estradiol (●) was assayed as a control

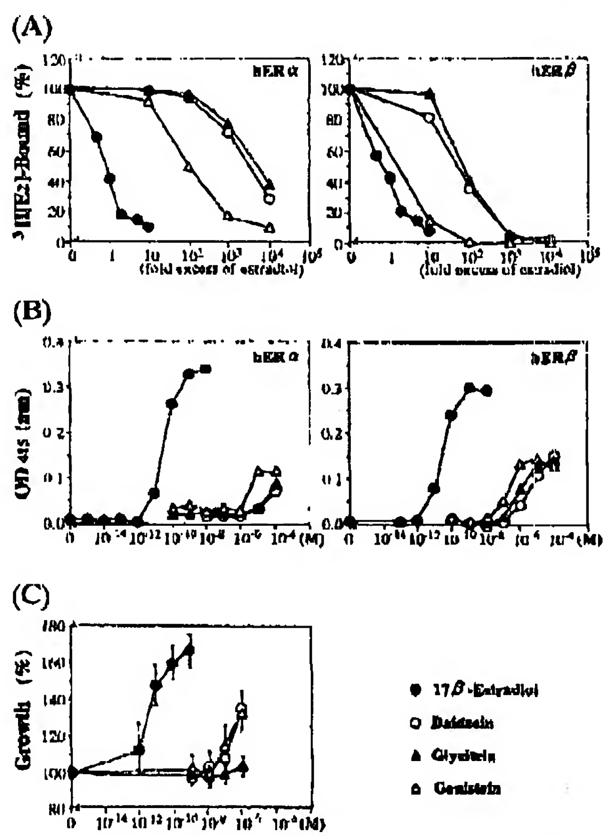

Fig. 5. The Same Assay as Fig. 3 with Daidzein (○), Glycitein (▲), and Genistein (△)

17 β-Estradiol (●) is a control

shown in Fig. 1 were examined and are shown in Figs. 4, 5, and 6. Figure 4 shows the results obtained for the glycosides: daidzin (DI), glycitin (GLI) and genistin (GI); Fig. 5 shows the results obtained for daidzein (DE), glycitein (GLE) and genistein (GE); and Fig. 6 shows the results obtained for equol (EQ), dihydroglycitein (DGL), and dihydrogenistein (DGE). The results show that glycosides bound poorly to both ER *α* and *β*, and induced transcription poorly. The aglycones generally bind to and induce transcription better with hER *β* than with hER *α*. Genistein is the strongest in binding. Though genistein binds ER *β* as strongly as 17 *β*-estradiol, it does not induce transcription as strongly as 17 *β*-estradiol or DES. Dihydrogenistein binds and induces transcription as efficiently as genistein. The binding of equol is similar to that of genistein and equol is the strongest among these compounds in inducing transcription, especially with hER *α*. The activity of daidzein is poor. Glycitein binds and induces transcription but the activities of glycitin derivatives are the poorest among these compounds.

The compounds that induce transcription generally stimulated the growth of MCF-7 cells. Genistin, a glycoside of genistein, however, stimulated the growth of cells better than genistein, even though genistin binds to the receptors more weakly and is less effective in inducing transcription than genistein.

DISCUSSION

Estrogens are critical to the functioning and maintenance of a diverse array of tissues and physiological systems in mammals. The physiological responses to estrogen are known to be mediated within specific tissues by at least two estrogen receptors (ERs), ER *α* and *β*. Studies of the tissue distributions and expression patterns of these receptors indicate that ER *α* has a broad expression pattern, whereas ER *β* has a more focused pattern, with high levels in the ovary, prostate, epididymis, lung and hypothalamus.[20,21] The effects of disruption of the ER *α* gene in ER *α* knockout mice include an absence of breast development in females and infertility caused by reproductive tract, gonadal and behavioral abnormalities in both sexes.[21–26] On the contrary, mice lacking ER *β* develop normally.[27] Recently, a mouse lacking both ER *α* and *β* was constructed.[28] Both sexes of this mouse are infertile, but they seem to grow normally and exhibit normal reproductive tract development.

Isoflavones are known to have estrogenic activity.[29] We are interested in their activities on ER *α* and *β*. Isoflavones contained in soybeans were digested by enteric bacteria. The estrogenic activities of the isolated digests were examined with respect to their binding to hER *α* and *β*, estrogen receptor-dependent transcriptional expression, and growth of MCF-7 cells.

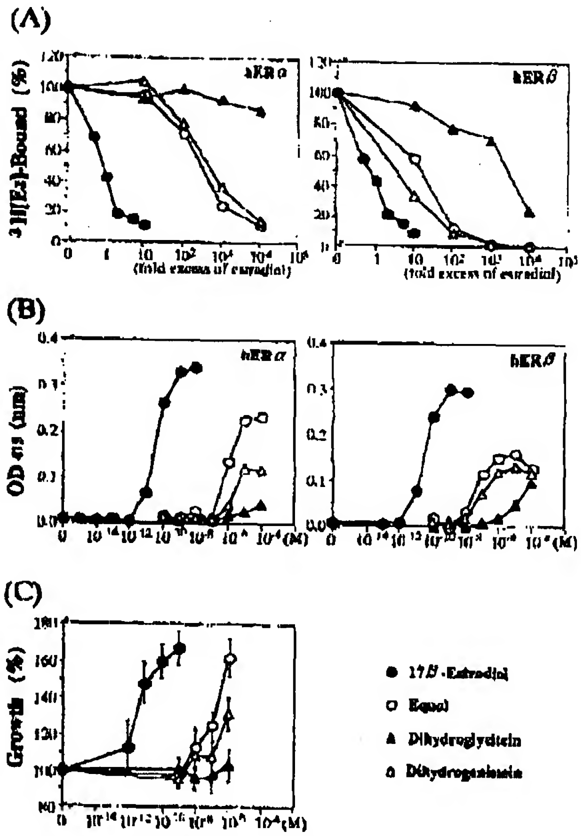

Fig. 6. The Same Assay as Fig. 3 with Equol (○), Dihydroglycitein (▲), and Dihydrogenistein (△)

17 β-estradiol (●) is a control.

All three glycosides (daidzin, glycitin, and genistin) bind poorly to these receptors and induce transcription poorly. Though genistin binds hER β, the affinity is weak compared with genistein (Figs. 4 and 5). Genistin, however, stimulated the growth of MCF-7 cells more strongly than genistein. The mechanism of growth stimulation of genistin must be different from that of genistein. Recently Simonocini *et al.* reported that they found a physiologically important non-nuclear estrogen-signalling pathway involving the direct interaction of ER α with PI(3)K.[30] Genistin might act through the signal transduction.

17 β-Estradiol and DES bind to both hER α and β with almost the same affinity. However, Bis A and NP, which have been suggested to be environmental endocrine disruptors,[18] and isoflavone derivatives bind more efficiently to hER β than α. The binding affinities of genistein, dihydrogenistein and equol to hER β are almost the same as the binding affinity of 17 β-estradiol.

Isoflavone derivatives generally induce receptor-dependent transcription and the induction is stronger with hER β than with α. Although these derivatives bind more strongly to hER β than α, the concentration required for the induction is almost the same with hER β as with hER α and is much higher than expected from the binding affinity. Among these derivatives, equol is especially strong at inducing transcription with hER α.

Genistein binds to hER β with almost the same efficiency as 17 β-estradiol, but the concentration required to induce transcription is 10^4 times greater for genistein than it is for 17 β-estradiol. Even if genistein bound as efficiently as 17 β-estradiol, the structural transformation of hER β induced by genistein would not be sufficient to facilitate the binding of a coactivator. The induction of transcription by ERs requires a coactivator.[10]

The E·F region of ER is the ligand-binding comain. The amino acid sequence of the E·F region of hER α is quite different from that of hER β in region F.[13,31,32] This difference is probably responsible for the difference of the binding affinities of the isoflavones for hER α and β.

Pike *et al.*[33] studied the structure of the ligand-binding domain of hER β in the presence of genistein. They found 3-OH of genistein corresponds to the 17-OH of 17 β-estradiol and 4′-OH of genistein corresponds to 6-OH of the sterol.

Epidemiological studies suggest that genistein and daidzein reduce the risk of breast and prostate cancers. Although our studies show that not only genistein and daidzein but also equol and glycitein stimulate the growth of MCF-7 cells, the concentrations of these compounds required for cell growth are much higher than the concentration of 17 β-estradiol that is needed. The much higher concentrations required for stimulating cell growth than for binding may explain why these compounds help to reduce the risk of cancer. The preferential expression of ER β in breast and prostate[34] and the preferential binding of isoflavones to ER β may explain why these compounds reduce the risk of cancers in these organs. This hypothesis could be tested by determining whether the incidence of breast cancer is reduced by isoflavones in mice lacking ER β (no reduction in breast cancer would be expected in such mice).

It has been reported that intake of isoflavone reduced the serum concentration of estradiol by feedback regulation, and that genistein inhibited tyrosine kinase, which is involved in the cell cycle.[35-37] These functions of isoflavone will also help to reduce the risk of cancer.

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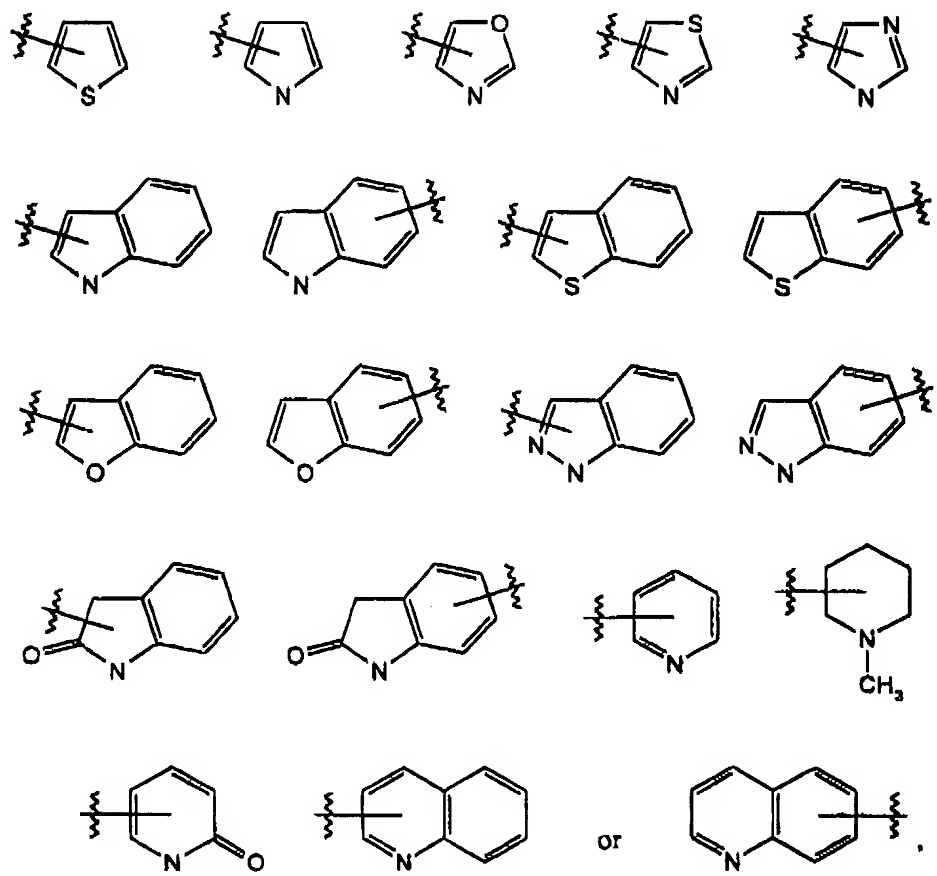
When L^1 is -C(=O)-, and R^2 is hydroxy or hydrogen, and R^3 is hydrogen, and R^4 is hydroxy, and R^5 is hydrogen, and R^6 is hydrogen, then R^1 is not para-phenol.

For purposes of this invention, "substituted" when used to modify a phenyl or a heteroatomic ring means such a ring substituted at one or more positions, independently, with $-R^a$, $-OR^a$, $-SR^a$, $-NR^aR^a$, $-NC(=O)R^a$, $-NS(=O)R^a$, $-NS(=O)_2R^a$, halogen, cyano, $-CF_3$, $-CO_2R^a$, $-C(=O)R^a$, $-C(=O)NHR^a$, nitro, $-S(=O)R^a$, or $-S(=O)_2R^a$.

Also, for purposes of this invention, "Het" means a substituted or unsubstituted one- or two-ring heterocycle selected from the following:



wherein the crossed bond represents that the heterocycle may be attached at any available position on the ring that it crosses.

Estrogen Receptor Binding Measurements

The ability of a compound to bind to ER was measured by its ability to compete for binding with the radio-labeled estrogen. [^{125}I]-16α–iodo–3,17β–estradiol (NEN. Cat.#NEX-144). The radio-ligand is hereafter referred to as [^{125}I]-estradiol.

ER-β (Gen Bank Accession #X99101) or ER-α (Gen Bank Accession #M12674) cDNAs were cloned into the expression vector pSG5 (Stratagene). transformed into *e. coli* strain DHαF'. and purified using anion-exchange resin columns (Qiagen Cat.#12125). Receptor protein was prepared by *in vitro* transcription and translation of these plasmids using the TNT T7 Quick-Coupled reticulocyte lysate system (Promega Cat.#L1170). Reticulocyte lysate (12.5 mL) was incubated for 90 min at 30 °C with 312.5 μg of ER-α and 625 μg of ER-β plasmids. Programmed lysate was then aliquotted and stored frozen at -80 °C.

Compounds were tested in duplicate at half-log concentrations ranging from 10 pM to 3 μM. Compounds were prepared as 1 mM stocks in DMSO, then diluted in the binding-assay buffer (in mM: 20 HEPES, 150 NaCl, 1 EDTA, 6 monothioglycerol and 10 Na_2MoO_4; 10% wt/vol glycerol. and pH = 7.9) to a series of three-fold concentrated, 20 μL aliquots in a 96-well plate. Receptor aliquots were thawed on ice. and appropriately diluted (see below) in binding assay buffer. Diluted receptor (30 μL/each) was added to each well. [^{125}I]-estradiol was diluted from the manufacturer's ethanol stock solution to a 900 pM working solution in binding-assay buffer. The final assay volume was 60 μL, consisting of 20 μL of a compound according to the instant invention, 30 μL of programmed reticulocyte lysate, and 10 μL of 900 pM [^{125}I]-estradiol. The final concentration of [^{125}I]-estradiol was 150 pM. Plates containing the final assay mixture were mixed on a shaker for 2 min and incubated overnight (~16 h) at 4 °C.

Receptor-bound and unbound radioligand was separated by filtration over sephadex columns. Columns (45 μL bed volume) were prepared by adding dry column media (Pharmacia Cat#G-25) to 96-well column templates (Millipore MultiScreen Plates Cat#MAHVN4510). Columns were then saturated with 300 μL of binding-assay buffer and stored at 4 °C. Prior to use. stored columns were spun for 10 minutes at 2000 RPM. then washed twice with 200 μL of fresh binding buffer. The binding-assay mixtures (50 μL/each) were then applied to the columns. and an additional elution volume of 35 μL was immediately applied to the column. Receptor-bound radioligand was then eluted from the column by

centrifugation for 10 minutes at 2000 RPM. A scintillation cocktail (145 μL) was added to the eluted radioligand/receptor complex, and radio-label was measured by liquid scintillation counting.

Non-specific binding was defined by competition with 150 nM diethylstilbesterol (DES). Binding affinities are expressed as K_i, calculated using the Cheng-Prushoff formula according to IC_{50} values generated by fitting the relationship of concentration to percent specific binding (SB) with the following equation:

$$\% SB = Maximum - (Maximum - Minimum)/(1+10^{(logIC50-log[Compound])})$$

In this assay, standard estrogen receptor ligands estradiol and DES were detected as high-affinity (K_i<1 nM), non-selective ligands of ER-β and ER-α.

The volume of receptor-programmed reticulocyte lysate to be added to the binding assay was determined independently from two measurements made on each batch of receptor prepared. First, K_is were determined for standard compounds using a series of dilutions of the receptor preparation. Scatchard analysis of ligand binding affinity was performed at the receptor dilutions that produced reported K_is for these compounds and an acceptable signal:noise ratio (~10). These experiments indicated a K_D for [^{125}I]-estradiol of 0.1-1 nM, and a B_{max} of 5-30 pmol.

Administration and Use

Compounds of the present invention are shown to have high selectivity for ER-β over ER-α, and may possess agonist activity on ER-β without undesired uterine effects. Thus, these compounds, and compositions containing them, may be used as therapeutic agents in the treatment of various CNS diseases related to ER-β, such as, for example, Alzheimer's disease.

The present invention also provides compositions comprising an effective amount of compounds of the present invention, including the nontoxic addition salts, amides and esters thereof, which may, serve to provide the above-recited therapeutic benefits. Such compositions may also be provided together with physiologically-tolerable liquid, gel or solid diluents, adjuvants and excipients. The compounds of the present invention may also be combined with other compounds known to be used as therapeutic agents for the above or other indications.

These compounds and compositions may be administered by qualified health care professionals to humans in a manner similar to other therapeutic agents and, additionally, to

other mammals for veterinary use, such as with domestic animals. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified. The active ingredient is often mixed with diluents or excipients which are physiologically tolerable and compatible with the active ingredient. Suitable diluents and excipients are, for example, water, saline, dextrose, glycerol, or the like, and combinations thereof. In addition, if desired the compositions may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, stabilizing or pH-buffering agents, and the like.

The compositions are conventionally administered parenterally, by injection, for example, either subcutaneously or intravenously. Additional formulations which are suitable for other modes of administration include suppositories, intranasal aerosols, and, in some cases, oral formulations. For suppositories, traditional binders and excipients may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained-release formulations, or powders.

The present compounds may be formulated into the compositions as neutral or salt forms. Pharmaceutically-acceptable nontoxic salts include the acid addition salts (formed with the free amino groups) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or organic acids such as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

In addition to the compounds of the present invention that display ER-β activity, compounds of the present invention can also be employed as intermediates in the synthesis of such useful compounds.

Synthesis

Compounds within the scope of the present invention may be synthesized chemically by means well known in the art. The following Examples are meant to show general synthetic schemes, which may be used to produce many different variations by employing various commercially-available starting materials. These Examples are meant only as guides on how to make some compounds within the scope of the invention, and should not be interpreted as limiting the scope of the invention.

Examples

Example 1 (Route A)

-(3-Bromo-4-hydroxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one

1,3,5-Trihydroxybenzaldehyde (1.01 g, 6.25 mmol) and 3-bromo-4-hydroxyphenylacetic acid (1.44 g, 6.25 mmol) were suspended in $POCl_3$ (4 mL). After 1 min, an exothermic reaction occured. The mixture was allowed to cool to room temperature. Zinc chloride (1M ether solution, 4.7 mmol) was added and the mixture was heated at 75 °C for 1 h. After cooling, the mixture was partitioned in ethyl acetate and 1M aqueous HCl. The organic layer was washed with brine and dried with $MgSO_4$. Purification on silica gel (MeOH/dichloromethane, gradient) afforded 1-(2,4,6-trihydroxyphenyl)-2-(3-bromo-4-hydroxyphenyl)ethanone (390 mg) as a tan solid.

To 1-(2,4,6-trihydroxyphenyl)-2-(3-bromo-4-hydroxyphenyl)ethanone (370 mg) in DMF (5 mL) under nitrogen was added BF_3-Et_2O (0.83 mL, 6.55 mmol) dropwise, followed by methanesulfonyl chloride (0.507 mL, 6.55 mmol). The mixture was stirred at room temperature for 10 min and heated at 55 °C for 30 min. After cooling, the mixture was partitioned in ethyl acetate / 1M aqueous HCl. The organic layer was washed with 1M HCl and brine, and purified by C_{18} HPLC to give the title compound (55 mg).

Example 2 (Compound No. 28; Route B)

3-(4-hydroxyphenyl)-7-hydroxy-4-methylcoumarin

A solution of 2,4-dihydroxyacetophenone (1.1 g, 7.24 mmol), 4-hydroxyphenylacetic acid (1.45 g, 9.5 mmol) and potassium acetate (0.9 g, 9.2 mmol) in acetic anhydride (10 mL) was heated under reflux for 18 h. After cooling, the mixture was poured into ice and water. The solid was filtered, washed with ether and dried under vacuum to give 3-(4-acetoxyphenyl)-7-acetoxy-4-methylcoumarin (1.83 g).

A suspension of 3-(4-acetoxyphenyl)-7-acetoxy-4-methylcoumarin (500 mg) in THF (10 mL) and 1N aqueous sodium hydroxide (10 mL) was stirred for 1 h. The mixture is acidified to pH = 1 with concentrated HCl and extracted with EtOAc / water. The organic layer was washed with brine and dried over $MgSO_4$. Evaporation of the solvent and trituration of the residue with ether gave the title compound (140 mg).

The HPLC conditions (HPLC 4.6 x 250 mm C_{18} 5 µm Vydax 218TP54 column, flow rate: 1.5 mL/min, acetonitrile/water 0.1% TFA linear gradient from 10:90 to 50:50 over 30 min. UV detection: 254 nm) are referred as conditions A.

The HPLC conditions (HPLC 2.1 x 30 mm C_{18} 3.5 µm Zorbax Rapid Resolution column, flow rate: 0.7 mL/min, water - 0.05% TFA for 0.5 min, then 90% aqueous acetonitrile/water 0.05% TFA linear gradient from 0:100 to 80:20 over 9.5 min, UV detection) are referred as conditions B.

The following compounds were prepared according to these routes, using the relevant starting materials.

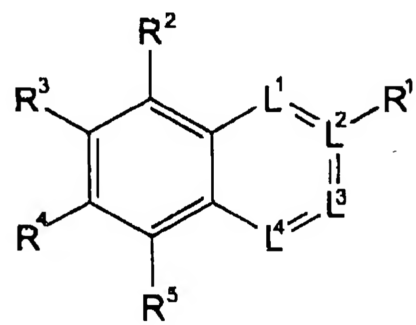


Table 1.

No.	L^1	L^2	L^3	L^4	R^1
1	C(=O)	=C-	=CR^6-	O	3,4-dihydroxyphenyl
2	C(=O)	=C-	=CR^6-	O	2-Cl-4-hydroxyphenyl
3	C(=O)	=C-	=CR^6-	O	2-Me-4-hydroxyphenyl
4	C(=O)	=C-	=CR^6-	O	3-F-4-hydroxyphenyl
5	C(=O)	=C-	=CR^6-	O	3-Cl-4-hydroxyphenyl
6	C(=O)	=C-	=CR^6-	O	3-Br-4-hydroxyphenyl
7	C(=O)	=C-	=CR^6-	O	3-allyl-4-hydroxyphenyl
8	C(=O)	=C-	=CR^6-	O	3-Pr-4-hydroxyphenyl

No.	L^1	L^2	L^3	L^4	R^1
9	C(=O)	=C-	$=CR^6-$	O	3-methoxy-4-hydroxyphenyl
10	C(=O)	=C-	$=CR^6-$	O	3.5-diMe-4-hydroxyphenyl
11	C(=O)	=C-	$=CR^6-$	O	4-fluorophenyl
12	C(=O)	=C-	$=CR^6-$	O	3.4-(OCH_2O)phenyl
13	C(=O)	=C-	$=CR^6-$	O	4-aminophenyl
14	C(=O)	=C-	$=CR^6-$	O	2-naphthyl
15	C(=O)	=C-	$=CR^6-$	O	3-hydroxyphenyl
16	C(=O)	=C-	$=CR^6-$	O	2-hydroxyphenyl
17	C(=O)	=C-	$=CR^6-$	O	2-thiophene
18	C(=O)	=C-	$=CR^6-$	O	3-thiophene
19	C(=O)	=C-	$=CR^6-$	O	2-quinolinyl
20	C(=O)	=C-	$=CR^6-$	O	4-bromophenyl
21	C(=O)	=C-	$=CR^6-$	O	4-chlorophenyl
22	C(=O)	=C-	$=CR^6-$	O	4-hydroxyphenyl
23	C(=O)	=C-	$=CR^6-$	O	4-hydroxyphenyl
24	C(=O)	=C-	$=CR^6-$	O	3-F-4-hydroxyphenyl
25	C(=O)	=C-	$=CR^6-$	O	4-hydroxyphenyl
26	C(=O)	-CH-	$-CHR^6-$	O	4-hydroxyphenyl
27	C(=O)	-CH-	$-CHR^6-$	CH_2	4-hydroxyphenyl
28	$=CR^6-$	=C-	C(=O)	O	4-hydroxyphenyl
29	$=CR^6-$	=C-	C(=O)	O	4-hydroxyphenyl
30	$=CR^6-$	=C-	C(=O)	O	4-hydroxyphenyl
31	$=CR^6-$	=C-	C(=O)	O	2-thiophene
32	C(=O)	=C-	$=CR^6-$	O	4-hydroxyphenyl
33	C(=O)	=C-	$=CR^6-$	O	2-F-phenyl
34	C(=O)	=C-	$=CR^6-$	O	phenyl
35	C(=O)	=C-	$=CR^6-$	O	phenyl
36	O	=C-	$=CR^6-$	C(=O)	4-hydroxyphenyl

No.	L^1	L^2	L^3	L^4	R^1
37	CH_2	-CH-	-CHR^6-	C(=O)	4-hydroxyphenyl
38	=CR^6-	=C-	C(=O)	O	4-hydroxyphenyl
39	=CR^6-	=C-	C(=O)	O	4-hydroxyphenyl
40	=CR^6-	=C-	C(=O)	O	4-hydroxyphenyl
41	=CR^6-	=C-	C(=O)	O	4-hydroxyphenyl
42	=CR^6-	=C-	C(=O)	O	4-Cl-phenyl
43	=CR^6-	=C-	C(=O)	O	4-hydroxyphenyl
44	C(=O)	=C-	=CR^6-	O	4-isopropoxyphenyl
45	C(=O)	-CH-	-CHR^6-	CH_2	3-Br-phenyl
46	CH_2	-CH-	-CHR^6-	O	4-hydroxyphenyl

(Continuation of Table 1)

No.	R^2	R^3	R^4	R^5	R^6
1	OH	H	OH	H	H
2	OH	H	OH	H	H
3	OH	H	OH	H	H
4	OH	H	OH	H	H
5	OH	H	OH	H	H
6	OH	H	OH	H	H
7	OH	H	OH	H	H
8	OH	H	OH	H	H
9	OH	H	OH	H	H
10	OH	H	OH	H	H
11	OH	H	OH	H	H
12	OH	H	OH	H	H
13	OH	H	OH	H	H
14	OH	H	OH	H	H
15	OH	H	OH	H	H
16	OH	H	OH	H	H

No.	R^2	R^3	R^4	R^5	R^6
17	OH	H	OH	H	H
18	OH	H	OH	H	H
19	OH	H	OH	H	H
20	OH	H	OH	H	H
21	OH	H	OH	H	H
22	OH	H	OMe	H	H
23	Me	H	OH	H	H
24	H	H	OH	H	H
25	H	H	OH	H	CF_3
26	OH	H	OH	H	H
27	OH	H	OH	H	H
28	H	H	OH	H	Me
29	H	H	OH	H	Et
30	H	H	H	H	H
31	H	H	OH	H	H
32	OH	H	OH	OMe	H
33	OH	H	OH	H	H
34	OH	H	OH	H	Ph
35	H	H	OH	H	Ph
36	H	H	OH	H	H
37	H	H	OH	H	H
38	H	H	OH	H	H
39	OH	H	OH	H	H
40	H	H	H	OH	H
41	H	OH	H	H	H
42	H	H	OH	H	Me
43	H	H	OH	Me	Me
44	H	H	OH	H	CF_3
45	H	H	OH	H	H

No.	R^2	R^3	R^4	R^5	R^6
46	H	H	OH	H	H

Table 2. Purification, Properties, and Synthetic Route

No.	HPLC min (method)	MS (MH^+)	ER-ß K_i nM	ER-α K_i nM	Synthetic Route
1			2.15	605	*
2	5.76 (B)	305 (^{35}Cl)	0.55	56	A
3	5.41 (B)	285	1.2	61	A
4	5.62 (B)	289	0.5	74	A
5	6.11 (B)	305 (^{35}Cl)	1.2	1100	A
6	25.6 (A)	349 (^{79}Br)	1.25	439	A
7	6.72 (B)	311	3.2	>3000	A
8	7.08 (B)	313	0.75	>3000	A
9			143	>3000	*
10	25.4 (A)	299	25	>3000	A
11	6.93 (B)	273	100	>3000	A
12			22	>3000	*
13			6	>3000	*
14	7.86 (B)	305	150	>3000	A
15	5.39 (B)	271	15	900	A
16	5.68 (B)	271	110	>3000	A
17	^1H NMR (DMSO-d_6): 12.59 (s, 1H), 10.99 (s, 1H), 8.88 (s, 1H), 7.63 (m, 2H), 7.14 (m, 1H), 6.44 (s, 1H), 6.27 (s, 1H).		3.3	>3000	A
18	^1H NMR (DMSO-d_6): 12.92 (s, 1H), 10.93 (s, 1H), 8.72 (s, 1H), 8.07 (s, 1H), 7.64 (m, 1H), 7.53 (m, 1H), 6.42 (s, 1H), 6.24 (s, 1H).		17	>3000	A
19	5.26 (B)	306	122	>3000	A
20	7.70 (B)	333 (^{79}Br)	25	>3000	A

No.	HPLC min (method)	MS (MH⁺)	ER-β K_i nM	ER-α K_i nM	Synthetic Route
21	7.55 (B)	289 (^{35}Cl)	42	>3000	A
22			50	>3000	*
23	5.20 (B)	269	0.5	200	A
24	4.91 (B)	273	3.3	>3000	A
25	6.07 (B)	323	10	321	Note a)
26			3.7	1000	*
27	5.43 (B)	271	5.7	3000	Note b)
28	^{1}H NMR (DMSO-d_6): 10.47 (m. 1H), 9.55 (m. 1H). 7.67 (d. 1H). 7.1-6.7 (m. 6H). 2.22 (m. 3H); MS: 269		12	322	B
29	5.57 (B)	283	4	80	B
30	6.01 (B)	239	140	>3000	B
31	^{1}H NMR (DMSO-d_6): 10.68 (s, 1H), 8.44 (s, 1H), 7.75 (m, 1H), 7.60 (m, 2H), 7.16 (m, 1H), 6.87 (dd, 1H), 6.81 (m, 1H); MS: 245		108	>3000	B
32			33	>3000	*
33	^{1}H NMR (DMSO d-6): 12.66 (s, 1H), 10.98 (s, 1H), 8.42 (s, 1H), 7.48 (m, 2H). 7.27 (m, 2H), 6.44 (d, 1H, J= 2.1 Hz), 6.26 (d, 1H, J= 2.1 Hz); MS: 273		50	>3000	A
34			9.5	95	*
35			19	50	*
36			0.33	88	*
37	^{1}H NMR (DMSO d-6): 9.61 (s, 1H), 9.52 (s, 1H). 7.26 (d, 1H. J= 2.7 Hz). 7.21-7.13 (m, 3H). 6.99 (dd, 1H, J= 8.1 Hz, J'= 2.7 Hz), 6.71 (d, 2H, J= 8.4 Hz), 3.26 (m, 1H), 3.07-2.80 (m. 3H). 2.64 (m, 1H); MS: 253 (M-H)⁻		0.73	75	Note c)

No.	HPLC min (method)	MS (MH$^+$)	ER-β K_i nM	ER-α K_i nM	Synthetic Route
38	^{1}H NMR (DMSO d-6): 10.52 (s, 1H), 9.64 (s, 1H), 8.03 (s, 1H), 7.55 (m, 3H), 6.85-6.70 (m, 4H); MS: 255		4.9	220	B
39	^{1}H NMR (DMSO d-6): 10.63 (s, 1H), 10.33 (s, 1H), 9.60 (s, 1H), 7.95 (s, 1H), 7.50 (d, 2H, J= 8.4 Hz), 6.80 (d, 2H, J= 8.4 Hz), 6.28 (s, 1H), 6.22 (s, 1H); MS: 271		79	>3000	B
40	^{1}H NMR (DMSO d-6): 10.18 (s, 1H), 9.73 (s, 1H), 8.08 (s, 1H), 7.60 (d, 2H, J= 8.4 Hz), 7.17 (m, 2H), 7.06 (m, 1H), 6.85 (d, 2H, J= 8.4 Hz); MS: 255		104	>3000	B
41	^{1}H NMR (DMSO d-6): 9.72 (s, 2H), 8.05 (s, 1H), 7.58 (d, 2H, J= 8.4 Hz), 7.25 (d, 1H, J= 8.7 Hz), 7.07 (d, 1H, J= 2.7 Hz), 7.00 (dd, 1H, J= 8.4 Hz, J'= 2.7 Hz), 6.84 (d, 2H, J= 8.4 Hz); MS: 255		4.6	3000	B
42	^{1}H NMR (DMSO d-6): 10.56 (s, 1H), 7.50 (d, 2H, J= 7.8 Hz), 7.42 (d, 1H, J= 8.7 Hz), 7.33 (d, 2H, J= 7.8 Hz), 6.84 (dd, 1H, J= 7.8 Hz, J'= 2.1 Hz), 6.75 (d, 1H, J= 2.1 Hz), 2.21 (s, 3H); MS: 287 (^{35}Cl)		51	>3000	B
43	^{1}H NMR (DMSO d-6): 10.36 (s, 1H), 9.55 (s, 1H), 7.49 (d, 1H, J= 9 Hz), 7.08 (d, 2H, J= 8.7 Hz), 6.87 (d, 1H, J= 9 Hz), 6.81 (d, 2H, J= 8.7 Hz), 2.21 (s, 3H), 2.19 (s, 3H); MS: 283		24	500	B
44	^{1}H NMR (DMSO d-6): 11.11 (s, 1H), 7.93 (d, 1H, J= 8.7 Hz), 7.16 (d, 2H, J= 8.4 Hz), 7.03-6.93 (m, 4H), 4.66 (m, 1H), 1.30 (d, 6H, J= 6Hz); MS: 365		118	3000	Note a)

No.	HPLC min (method)	MS (MH^+)	ER-ß K_i nM	ER-α K_i nM	Synthetic Route
45	^1H NMR (DMSO d-6): 10.39 (s, 1H), 7.78 (d, 1H, J= 8.4 Hz), 7.42 (m, 2H), 7.28 (t, 1H, J= 7.8 Hz), 7.19 (d, 1H, J= 7.8 Hz), 6.75 (dd, 1H, J= 8.4 Hz, J'= 2.4 Hz), 6.69 (d, 1H, J= 2.4 Hz), 3.86 (m, 1H), 3.00 (m, 1H), 2.85 (m, 1H), 2.4-2.1 (m, 2H); MS: 317 (^{79}Br)		116	3000	Note b)
46			2	155	*

* compound is commercially available.

Note a): Prepared according to method A; the cyclization step was done using trifluoroacetic anhydride according to J. Med. Chem. 1992, 35, 3519.

Note b): Prepared by cyclization of the corresponding 2,4-diarylbutyric acid with $POCl_3$, and subsequent demethylation of the methoxy ethers according to the method developed in J. Org. Chem. 1946, 11, 34.

Note c): Prepared according to Aust. J. Chem. 1978, 31, 1011.

CLAIMS:

1. A method for treating a disease associated with the estrogen receptor-β, comprising the step of administering a therapeutically-effective amount of a compound that satisfies the equation:

$$(K_{i\alpha A}/Ki_{\beta A})/(K_{i\alpha E}/K_{i\beta E}) > 1,$$

wherein

$K_{i\alpha A}$ is the K_i value for the agonist in ER-α;

$K_{i\beta A}$ is the K_i value for the agonist in ER-β;

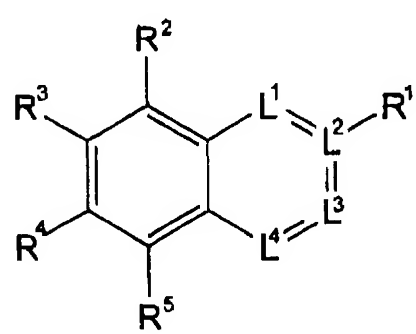
$K_{i\alpha E}$ is the K_i value for estrogen in ER-α; and

$K_{i\beta E}$ is the K_i value for estrogen in ER-β.

2. The method according to Claim 1, wherein the compound satisfies the equation: $(K_{i\alpha A}/Ki_{\beta A})/(K_{i\alpha E}/K_{i\beta E}) > 100$.

3. The method according to Claim 2, wherein the disease to be treated is selected from the group consisting of Alzheimer's disease, anxiety disorders, depressive disorders, osteoporosis, cardiovascular disease, rheumatoid arthritis and prostate cancer.

4. The method according to Claim 3, wherein the compound has the formula:



wherein:

L^1 is -C(=O)-, =C(R^6)-, -CH(R^6)-, O, S, or NR^7;

L^2 is =C- or -CH-;

L^3 is $=C(R^6)$-, $-CH(R^6)$- or $-C(=O)$-;

L^4 is $-C(=O)$-, CH_2, O, S, or NR^a;

wherein:

when L^1 is $-C(=O)$-, L^4 is CH_2, O, S, or NR^a;

when L^4 is $-C(=O)$-, L^1 is CH_2, O, S, or NR^a;

when L^3 is $-C(=O)$-, L^1 is $=C(R^6)$- or $-CH(R^6)$-, and L^4 is O or NR^a

when L^1 is $=C(R^6)$-, L^2 is $=C$-;

when L^1 is $-CH(R^6)$-, L^2 is $-CH$-;

when L^3 is $=C(R^6)$-, L^2 is $=C$-; and

when L^3 is $-CH(R^6)$-, L^2 is $-CH$-;

R^a is, independently, at each occurrence, H or $(C_1$-$C_5)$alkyl;

R^1 is phenyl, substituted phenyl or Het;

R^2, R^3, R^4 and R^5 are independently selected from the group consisting of $-R^a$, $-OR^a$, $-SR^a$, $-NR^aR^a$, $-NC(=O)R^a$, $-NS(=O)R^a$, $-NS(=O)_2R^a$, halogen, cyano, $-CF_3$, $-CO_2R^a$, $-C(=O)R^a$, $-C(=O)NHR^a$, nitro, $-S(=O)R^a$ and $-S(=O)_2R^a$;

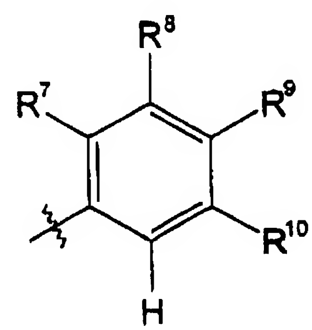
R^6 is H, $(C_1$-$C_5)$alkyl, phenyl or CF_3; and

wherein, when L^1 is $-C(=O)$-, and R^2 is hydroxy or hydrogen, and R^3 is hydrogen, and R^4 is hydroxy, and R^5 is hydrogen, and R^6 is hydrogen then R^1 is not para-phenol; and any pharmaceutically-acceptable salt thereof.

5. The method according to Claim 4, wherein R^1 is Het.

6. The method according to Claim 4, wherein:

R^1 has the structure:



wherein:

R^7 is H, Cl or methyl;

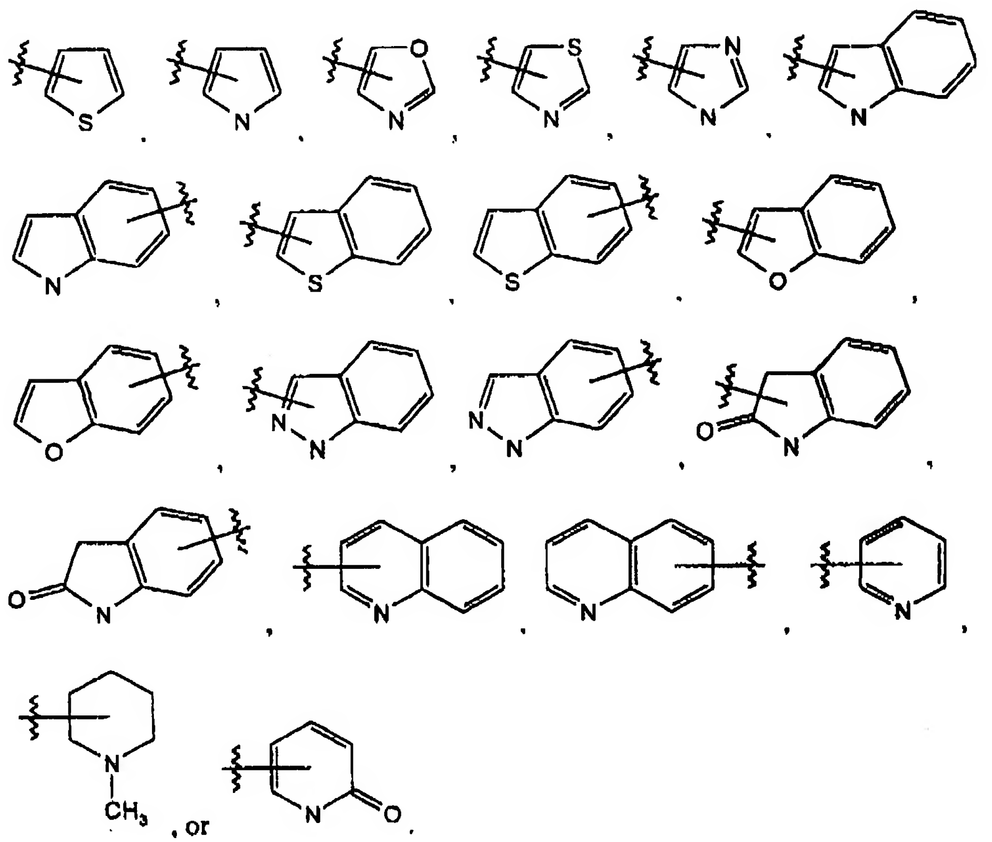
R^8 is Br, Cl, F, R^a, OR^a or allyl;

R^9 is H, OH, NH_2, Br or Cl; and

R^{10} is H or methyl; or

R^8 and R^9 combine to form $-OCH_2O-$; or

R^1 is a substituted or unsubstituted version of one of the following:



7. The method according to any one of Claims 6, wherein the disease is Alzheimer's disease or depressive disorders.

8. The method according to Claim 6 wherein R^2, R^3, R^4 and R^5 are independently selected from the group consisting of R^a, OR^a, $NR^a{}_2$, $NC(=O)R^a$, CF_3 and halogen.

9. The method according to Claim 8 wherein:

R^2 is hydroxyl or hydrogen;

R^3 is hydrogen or methyl;

R^4 is hydroxyl or hydrogen; and

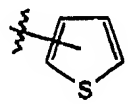
R^5 is hydrogen or hydroxyl.

10. The method according to Claim 8 wherein L^4 is -C(=O)-.

11. The method according to Claim 8 wherein L^3 is -C(=O)-.

12. The method according to Claim 8 wherein L^1 is -C(=O)-.

13. The method according to Claim 9 wherein R^1 is an unsubstituted version of



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Interaction of Phytoestrogens with Estrogen Receptors α and β

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The human estrogen receptor (hER) exists as two subtypes, hER α and hER β, that differ in the C-terminal ligand-binding domain and in the N-terminal transactivation domain. In this study, we investigated the estrogenic activities of soy isoflavones after digestion with enteric bacteria in competition binding assays with hER α or hER β protein, and in a gene expression assay using a yeast system. The estrogenic activities of these isoflavones were also investigated by the growth of MCF-7 breast cancer cells.

Isoflavone glycoside binds weakly to both receptors and estrogen receptor-dependent transcriptional expression is poor. The aglycones bind more strongly to hER β than to hER α. The binding affinities of genistein, dihydrogenistein and equol are comparable to the binding affinity of 17 β-estradiol. Equol induces transcription most strongly with hER α and hER β. The concentration required for maximal gene expression is much higher than expected from the binding affinities of the compounds, and the maximal activity induced by these compounds is about half the activity of 17 β-estradiol. Although genistin binds more weakly to the receptors and induces transcription less than does genistein, it stimulates the growth of MCF-7 cells more strongly than does genistein.

Key words isoflavone; human estrogen receptor (hER) α, β; isoflavone binding to hER; hER-dependent gene expression; hER-dependent MCF-7 cell growth

Estrogens play important hormonal roles in all vertebrates. Animal estrogens are exclusively steroidal compounds, and the principal physiological estrogen in most species is 17 β-estradiol. Many plants produce compounds that possess estrogenic activity in animals and are thus called phytoestrogens.

Among the foods consumed by humans, soybeans contain the highest concentration of isoflavones. These soy isoflavones (e.g., daidzin, genistin and glycitin) may have some health-enhancing properties such as prevention of certain cancers,[1] lowering the risk of cardiovascular diseases,[2] and improvement of bone health.[3] The estrogenic activities of these isoflavones may play an important role in their health-enhancing properties. Soy isoflavones have been reported to bind to estrogen receptors and prevent cell growth in breast cancer cells.[4,5]

We have systematically examined the metabolism of soy isoflavones by enteric bacteria and identified these metabolites.[6]

Two estrogen receptors (ERs) have been identified to date[8] and the physiological responses to estrogen are known to be mediated within specific tissues by at least these two receptors. The ERs are a 3A member of the nuclear hormone receptor family and act as ligand-activated nuclear transcription factors.[9]

In this paper, we examined the estrogenic activities of the isoflavone metabolites by (A) their binding to hER α and β, (B) their effect on estrogen receptor-dependent transcriptional expression, and (C) their effect on the growth of MCF-7 cells, which requires estrogen for growth.[10,11]

MATERIALS AND METHODS

Chemicals 17 β-Estradiol, Diethylstilbestrol (DES), Bisphenol A (Bis A) and Nonylphenol (NP) were purchased from Sigma Chemical Co. [2,4,6,7-^{3}H(N)]-17 β-Estradiol (72 Ci/mmol) was purchased from Dai-Ichi Pure Chemicals Co., Ltd. MCF-7 cells were purchased from Dainippon Pharmaceutical Co., Ltd.

Isoflavones Soybean isoflavones were digested by enteric bacteria and the structures of these digests were determined and isolated as reported.[6] The products, which are examined in this paper, are shown in Fig. 1.

Preparation of the Extract of Human Estrogen Receptor α and β hER α cDNA was isolated from pBacPAK9-HEG0 kindly supplied by S. Kato[12] by digestion with *Bam*HI and *Xho*I. hER β cDNA was isolated from pGEX-4T-2-hER β[13] by digestion with *Bam*HI and *Xho*I. These fragments were ligated into the *Bam*HI-*Xho*I sites of the baculovirus donor vector pFastBac 1 (Life Technologies, Gaithersburg, MD, U.S.A.). Recombinant baculoviruses were generated using the BAC-TO-BAC expression system (Life Technologies) in accordance with the manufacturer's instructions. The recombinant baculoviruses were amplified and used to infect Sf21 cells (Clontech, Palo Alto, CA, U.S.A.). Infected cells were incubated at 28 °C and harvested 72 h post infection by centrifugation. The cells were suspended in buffer containing 40 mM Tris–HCl, pH 7.4, 0.5 mM EDTA, 0.2 M KCl, 10% glycerol, 1 mM DTT, and 1 mM PMSF. The extracts were prepared by sonication (10 s×2). The supernatants of the extracts after centrifugation (15000 rpm×10 min) contained *ca.* 6 mg/ml protein and were used as hER α and β. The concentrations of hER α and β were measured using purified hER α and β purchased from Takara Shuzo

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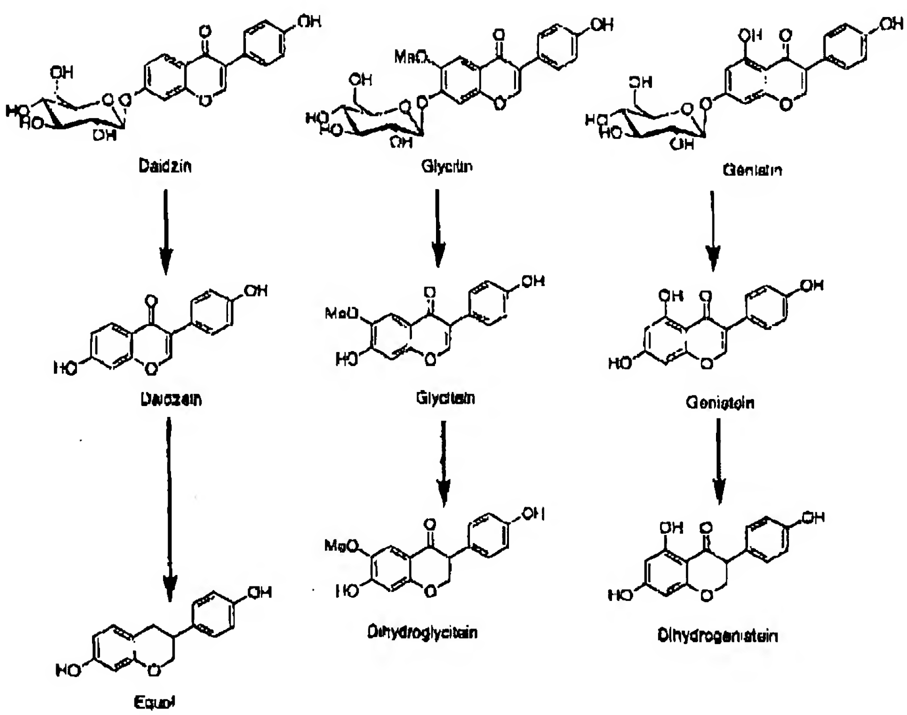

Fig. 1. Structures of Metabolites Obtained by the Digestion of Soy Isoflavones by Enteric Bacteria

Co., Ltd. as a standard. Their concentrations were 0.6% and 0.3% of the total protein, respectively. These receptors were stable at −80 °C for several months.

Competition Assay of Isoflavone Estrogen binding to hER α or β was determined by incubation of 250 μl of reaction mixture of TKE (20 mM Tris–HCl, pH 7.4, 1 mM EDTA, 20 mM KCl) containing 5 μl hER α or β with 2.5 pmol [^{3}H]-17 β-estradiol at 0 °C (for 16 h) in the presence of various concentrations of isoflavones. Isoflavones were stable under this incubation condition. Free and bound ligands were separated by addition of an equal volume of dextran-coated charcoal (0.5% activated charcoal and 0.05% dextran) in TKE. Samples were treated for 5 min on ice with periodic mixing and centrifuged at 15000 rpm for 1 min. Aliquots of the supernatant (300 μl) were used for scintillation counting.

Construction of Yeast Strain Carrying Full-Length hER α or β *Saccharomyces cerevisiae* Y190 (*MATa, ura3-52, his3-D200, ade2-101, trp1-901, leu2-3, 112, gal4Dgal80D, URA3::: GAL-lacZ, cyhr2, LYS2::: GAL-HIS3*) which carries pGBT9-ratER and pGAD424-*hTIF2* was kindly supplied by Nishikawa.[14] We substituted hER α or hER β for ratER. pGBT9-ratERLBD was digested with *Eco*RI, and then the cleaved open ends were treated with S1 nuclease. The digest was further digested with *Bam*HI followed by treatment with a Klenow fragment and closed by ligation. The plasmid was redigested with *Bam*HI and *Sal*I. pGBT9-hER α or pGBT9-hER β was prepared by inserting a fragment containing full-length hER α or hER β obtained by the digestion of pBacPAK9 HEG0 or pGEX4T-2-ER β at *Bam*HI and *Xho*I sites into the sites obtained by the digestion of pGBT9 by *Bam*HI and *Sal*I (Fig. 2).

Estrogen Receptor-Dependent Transcriptional Expression Induced by Isoflavone The effect of isoflavones on the estrogen receptor-dependent transcription of β-galactosidase in yeast was examined following the methods described by Nishikawa and his colleagues.[14] Yeast cells carrying hER α or β were constructed as described by these same authors.[14]

Growth of MCF-7 Cells MCF-7 cells were grown in phenol red-free DMEM (Gibco BRL, Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, U.S.A.), penicillin and streptomycin (Gibco BRL, Grand Island, NY, U.S.A.). Cells were grown as a monolayer under these conditions. Cells were harvested as needed for use in experimental trials by trypsinization (0.05% trypsin, 0.53 mM EDTA-4Na; Gibco) to yield a suspension of cells for plating in 96-well tissue-culture plates (Falcon; Becton Dickinson, Franklin Lakes, NJ, U.S.A.). Cells were plated at a concentration of 2×10^4 cells well in phenol red-free DMEM supplemented with 5% heat-inactivated dextran charcoal-stripped FBS (Hyclone, Logan, UT, U.S.A.) for 24 h prior to the addition of phytoestrogen. The growth of the cells was measured by a sulforhodamine B (SRB) assay[15] after 5 d incubation.

RESULTS

Estrogenic Activities of Isoflavones DES, Bis A, and NP are known to bind ER, induce transcription and stimulate the growth of MCF-7 cells.[16–18] We used these compounds as controls. The results are shown in Fig. 3. DES binds both hER α and β almost as strongly as 17 β-estradiol. Bis A and NP bind hER β better than α. The concentration required to bind 50% is about 10^3 times greater for Bis A or NP than it is for 17 β-estradiol. Tamoxifen which is known as an estrogen

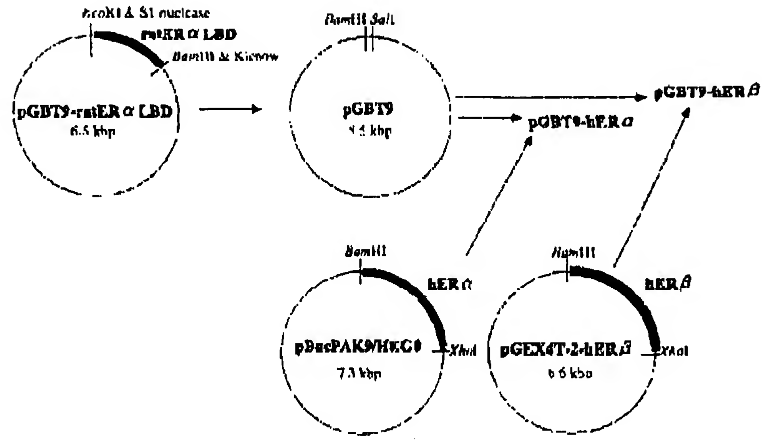

Fig. 2. Construction of pGBT9-hER α and pGBT9-hER β

hER α fragment was obtained by the digestion of pBacPAK9/HEG0 by BamHI and XhoI. This fragment was ligated with the fragment obtained by digesting pGBT9 by BamHI and SalI to prepare pGBT9-hER α. The hER β fragment was obtained by digesting pGEX4T-2-hER β by BamHI and XhoI. This fragment was ligated with the fragment obtained by digesting pGBT9 by BamHI and SalI to prepare pGBT9-hER β.

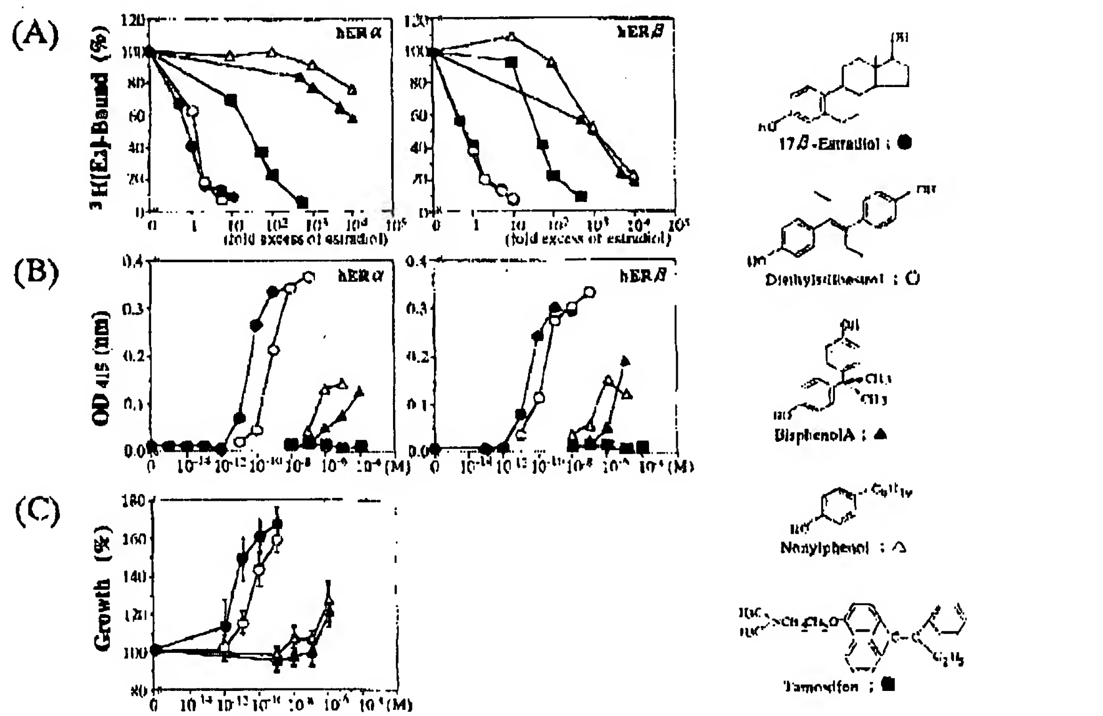

Fig. 3. Assays of (A) Binding of Estrogenic Compounds to hERs, (B) Estrogen Receptor-Dependent Transcriptional Expression, and (C) Growth of MCF-7 Cells

●, 17 β-estradiol; ○, DES; ▲, Bis A; △, NP; ■, tamoxifen. Binding to the ERs was examined by competition as described in Materials and Methods. Estrogen receptor-dependent β-galactosidase induction was measured by the increase of OD_{415nm} by p-nitrophenol produced by the digestion of p-nitrophenyl-β-D-galactoside. The growth of MCF-7 cells was measured by an SRB assay and the results are shown as percent increase. Although we have obtained fairly constant results concerning the binding to the ERs and the induction of β-galactosidase, the results of the growth of MCF-7 cells varied. We have done at least 10 experiments of the growth of MCF-7 cells with each compound and the average is shown. The bar at each point is the standard deviation.

antagonist[10] binds both hER α and β but does not induce transcription. Testosterone did not bind to these receptors confirming their specificity. We also confirmed that 17 β-estradiol did not bind to the cell extract prepared from the cells infected by vector baculovirus (data not shown).

In the experiments of estrogen receptor-dependent transcriptional expression, DES induces as efficiently as 17 β-estradiol. The concentration required for the maximal induction is about 10^4 times greater for Bis A or NP than it is for 17 β-estradiol. These drugs stimulate the growth of MCF-7 cells as shown in Fig. 3. We confirmed that 17 β-estradiol did not induce transcription with the cells carrying vector plasmid pGBT9 (data not shown).

The estrogenic activities of the isoflavone derivatives

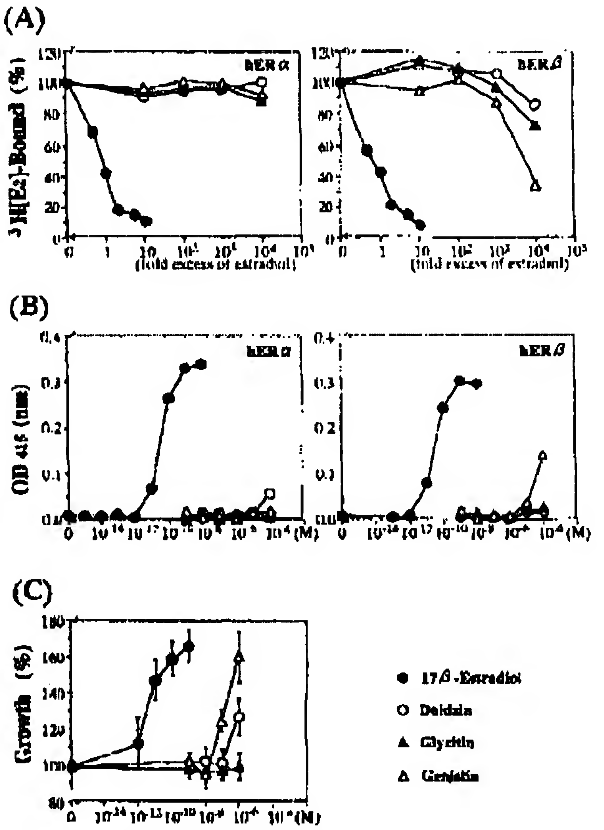

Fig. 4. The Same Assay as Fig. 3 with Daidzin (○), Glycitin (▲), and Genistin (△)

17 β-Estradiol (●) was assayed as a control

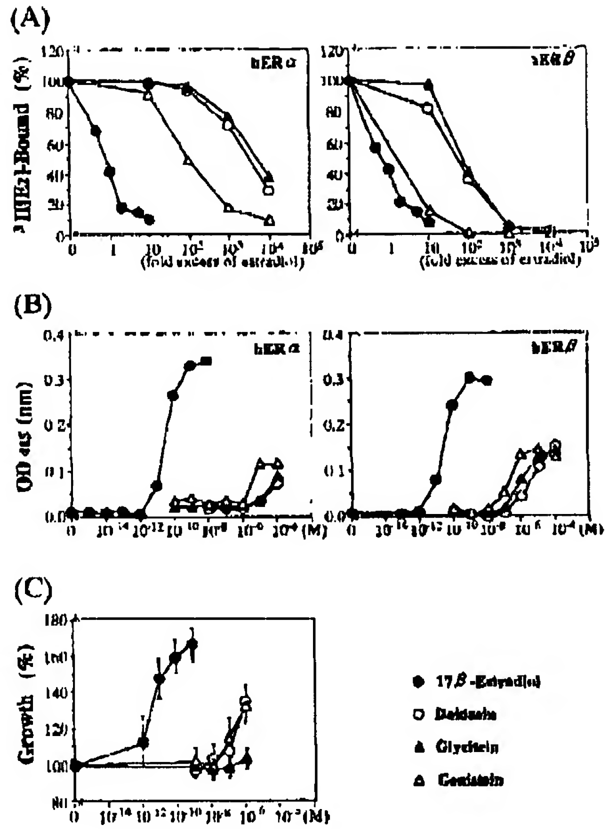

Fig. 5. The Same Assay as Fig. 3 with Daidzein (○), Glycitein (▲), and Genistein (△)

17 β-Estradiol (●) is a control

shown in Fig. 1 were examined and are shown in Figs. 4, 5, and 6. Figure 4 shows the results obtained for the glycosides: daidzin (DI), glycitin (GLI) and genistin (GI); Fig. 5 shows the results obtained for daidzein (DE), glycitein (GLE) and genistein (GE); and Fig. 6 shows the results obtained for equol (EQ), dihydroglycitein (DGL), and dihydrogenistein (DGE). The results show that glycosides bound poorly to both ER *α* and *β*, and induced transcription poorly. The aglycones generally bind to and induce transcription better with hER *β* than with hER *α*. Genistein is the strongest in binding. Though genistein binds ER *β* as strongly as 17 *β*-estradiol, it does not induce transcription as strongly as 17 *β*-estradiol or DES. Dihydrogenistein binds and induces transcription as efficiently as genistein. The binding of equol is similar to that of genistein and equol is the strongest among these compounds in inducing transcription, especially with hER *α*. The activity of daidzein is poor. Glycitein binds and induces transcription but the activities of glycitin derivatives are the poorest among these compounds.

The compounds that induce transcription generally stimulated the growth of MCF-7 cells. Genistin, a glycoside of genistein, however, stimulated the growth of cells better than genistein, even though genistin binds to the receptors more weakly and is less effective in inducing transcription than genistein.

DISCUSSION

Estrogens are critical to the functioning and maintenance of a diverse array of tissues and physiological systems in mammals. The physiological responses to estrogen are known to be mediated within specific tissues by at least two estrogen receptors (ERs), ER *α* and *β*. Studies of the tissue distributions and expression patterns of these receptors indicate that ER *α* has a broad expression pattern, whereas ER *β* has a more focused pattern, with high levels in the ovary, prostate, epididymis, lung and hypothalamus.[20,21] The effects of disruption of the ER *α* gene in ER *α* knockout mice include an absence of breast development in females and infertility caused by reproductive tract, gonadal and behavioral abnormalities in both sexes.[21–26] On the contrary, mice lacking ER *β* develop normally.[27] Recently, a mouse lacking both ER *α* and *β* was constructed.[28] Both sexes of this mouse are infertile, but they seem to grow normally and exhibit normal reproductive tract development.

Isoflavones are known to have estrogenic activity.[29] We are interested in their activities on ER *α* and *β*. Isoflavones contained in soybeans were digested by enteric bacteria. The estrogenic activities of the isolated digests were examined with respect to their binding to hER *α* and *β*, estrogen receptor-dependent transcriptional expression, and growth of MCF-7 cells.

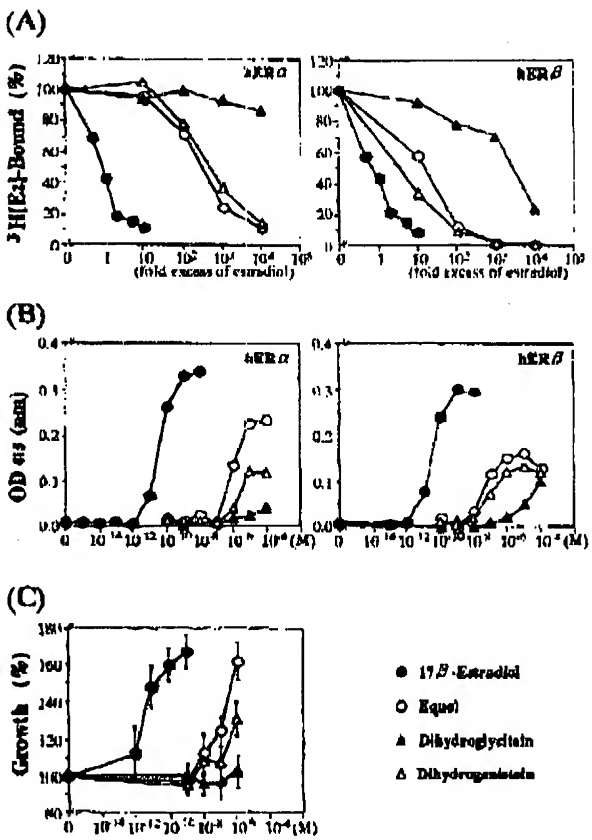

Fig. 6. The Same Assay as Fig. 3 with Equol (○), Dihydroglycitein (▲), and Dihydrogenistein (△)
17 β-estradiol (●) is a control.

All three glycosides (daidzin, glycitin, and genistin) bind poorly to these receptors and induce transcription poorly. Though genistin binds hER β, the affinity is weak compared with genistein (Figs. 4 and 5). Genistin, however, stimulated the growth of MCF-7 cells more strongly than genistein. The mechanism of growth stimulation of genistin must be different from that of genistein. Recently Simoncini *et al.* reported that they found a physiologically important non-nuclear estrogen-signalling pathway involving the direct interaction of ER α with PI(3)K.[30] Genistin might act through the signal transduction.

17 β-Estradiol and DES bind to both hER α and β with almost the same affinity. However, Bis A and NP, which have been suggested to be environmental endocrine disruptors,[18] and isoflavone derivatives bind more efficiently to hER β than α. The binding affinities of genistein, dihydrogenistein and equol to hER β are almost the same as the binding affinity of 17 β-estradiol.

Isoflavone derivatives generally induce receptor-dependent transcription and the induction is stronger with hER β than with α. Although these derivatives bind more strongly to hER β than α, the concentration required for the induction is almost the same with hER β as with hER α and is much higher than expected from the binding affinity. Among these derivatives, equol is especially strong at inducing transcription with hER α.

Genistein binds to hER β with almost the same efficiency as 17 β-estradiol, but the concentration required to induce transcription is 10^4 times greater for genistein than it is for 17 β-estradiol. Even if genistein bound as efficiently as 17 β-estradiol, the structural transformation of hER β induced by genistein would not be sufficient to facilitate the binding of a coactivator. The induction of transcription by ERs requires a coactivator.[16]

The E F region of ER is the ligand-binding domain. The amino acid sequence of the E F region of hER α is quite different from that of hER β in region F.[13,31,32] This difference is probably responsible for the difference of the binding affinities of the isoflavones for hER α and β.

Pike *et al.*[33] studied the structure of the ligand-binding domain of hER β in the presence of genistein. They found 3-OH of genistein corresponds to the 17-OH of 17 β-estradiol and 4'-OH of genistein corresponds to 6-OH of the sterol.

Epidemiological studies suggest that genistein and daidzein reduce the risk of breast and prostate cancers. Although our studies show that not only genistein and daidzein but also equol and glycitein stimulate the growth of MCF-7 cells, the concentrations of these compounds required for cell growth are much higher than the concentration of 17 β-estradiol that is needed. The much higher concentrations required for stimulating cell growth than for binding may explain why these compounds help to reduce the risk of cancer. The preferential expression of ER β in breast and prostate[34] and the preferential binding of isoflavones to ER β may explain why these compounds reduce the risk of cancers in these organs. This hypothesis could be tested by determining whether the incidence of breast cancer is reduced by isoflavones in mice lacking ER β (no reduction in breast cancer would be expected in such mice).

It has been reported that intake of isoflavone reduced the serum concentration of estradiol by feedback regulation, and that genistein inhibited tyrosine kinase, which is involved in the cell cycle.[35–37] These functions of isoflavone will also help to reduce the risk of cancer.

Acknowledgements We are grateful to Shigeaki Kato for kindly giving us plasmid pBacPAK9/HEG0 and to Tsutomu Nishihara for kindly giving us *Saccharomyces cerevisiae* Y190 which carries pGBT9-ratER and pGAD424-*hTIF2*. The experiment was partially supported by a grant from the Ministry of Health and Welfare of Japan.

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